THE EFFECTS OF A COMBINATION OF TWO RECEPTOR TYROSINE KINASE INHIBITORS ON TWO OVARIAN CANCER CELL LINES

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By
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Preface

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Abbreviations

Akt	Protein Kinase B
BSA	Bovine serum albumin
Canertinib	EGFR inhibitor
c-MET	Mesenchymal epithelial transition factor
Crystal violet	Tris (4-(dimethylamino) phenyl) methylium chloride
DGGE	Denaturing gradient gel electrophoresis
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-related kinase
FAK	Focal Adhesion Kinase
FBS	Foetal bovine serum
FISH	Fluorescence in situ hybridization
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HER-2/neu	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IL6	Interleukin-6
LPA	Lysophosphatidic acid
MEM	Minimum essential medium
MMP	Matrix metalloproteinase
NSCLC	Non-small cell lung cancer
OVCAR-5	Human ovarian adenocarcinoma cell line
p-Akt	Phosphorylated Protein Kinase B
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
p-EGFR	Phosphorylated Epidermal growth factor receptor
p-ERK	Phosphorylated Extracellular signal-related kinase

p-HER-2/neu	Phosphorylated Human epidermal growth factor receptor 2
PI3K	Phospho inositide-3-kinase
p-MET	Phosphorylated Mesenchymal epithelial transition factor
poly-HEMA	Poly-hydroxy ethyl methacrylate
PVDF	Polyvinyl difluoride
RIPA	Radio immuno precipitation assay
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEM	Standard error of the mean
TBS-T	Tris-buffered saline and Tween20
10% APS	10% Ammonium per sulfate

This thesis has contributed to the following:

Thesis publication

Hassan Wafaa, Chitcholtan Kenny, Sykes Peter, Garrill Ashley "A Combination of Two Receptor Tyrosine Kinase Inhibitors, Canertinib and PHA665752 Compromises Ovarian Cancer Cell Growth in 3D Cell Models" Oncol Ther 2016; 4(2): 257–274.

Other publications

Hogg Simon, Chitcholtan Kenny, **Hassan Wafaa**, Sykes Peter, Garrill Ashley "Resveratrol, Acetyl-Resveratrol, and Polydatin Exhibit Antigrowth Activity against 3D Cell Aggregates of the SKOV-3 and OVCAR-8 Ovarian Cancer Cell Lines" Obstetrics and Gynecology International 2015; 14pages.

Poster presentations

Queenstown research week 2013 "Targeting growth factor receptors in advanced ovarian cancer" **Wafaa Hassan**, Ashley Garrill, Kenny Chitcholtan, Biology dept. UC, Obstetrics and Gynaecology dept. Otago University-Christchurch.

NZSO conference 2015 "Combination of EGFR/HER-2 And c-MET Inhibitors Inhibit Growth and Adhesion In 3D Cell Clusters of Ovarian Cancer Cells" **Wafaa Hassan**, Kenny Chitcholtan, Peter Sykes, Ashley Garrill, Biological Sciences, University of Canterbury, Department of Obstetrics and Gynaecology, University of Otago-Christchurch, New Zealand.

Abstract

Ovarian cancer is a leading cause of mortality in gynaecological cancer in New Zealand and worldwide. New targeted approaches are essential to improve the survival and quality of life for patients with this disease. In advanced ovarian cancer, malignant cells shed off the ruptured ovarian tumour into the peritoneum forming 3D cellular aggregates in order to survive and establish secondary growth on the abdominal wall of internal organs.

Receptor tyrosine kinases including EGFR, HER-2 and c-MET are frequently overexpressed in ovarian cancer cells and have been linked with poor prognosis and low survival in ovarian cancer patients. The ability of ovarian cancer cells to utilize these receptors for survival and metastasis suggests an important role for co-activation and highlights a need for a better understanding of their role in cancer progression and the possibility of combined drug targeting for a better patient outcome.

In this thesis, an *in vitro* study of 3D cellular clusters and compact aggregates of two ovarian cancer cell lines (OVCAR-5 and SKOV-3) and their response to a combination of two tyrosine kinase inhibitors (TKI), canertinib (a pan EGFR inhibitor) and PHA665752 (a c-MET inhibitor) was investigated. The receptors were activated with their specific ligands EGF and HGF. The two cell lines were chosen on the basis of their molecular subtypes.

The inhibitor combination reduced the total expression and phosphorylation of the receptors as well as the downstream signalling molecules Akt and ERK. This then affected cell growth. Both inhibitors reduced cellular adhesion to collagen gel matrix. In an attempt to mimic the *in vivo* environment of the cancer cells the effect of ascitic fluids from advanced ovarian cancer patients on inhibitor efficacy was tested. The ascitic fluid was found to reduce the effect of the inhibitors. It is suggested that a component in the ascetic fluid may be preventing the inhibitors from entering the cells possibly through binding with serum.

CHAPTER ONE

INTRODUCTION

1.1 Ovarian cancer-Overview

Ovarian cancer is one of the most lethal gynaecological cancers [1]. It is the eighth most common type of malignancy [2, 3] and the fifth cause of cancer related mortality in females worldwide [4, 5]. According to a recent statistics report in the USA, approximately 22,280 new cases of ovarian cancers were diagnosed of which 14,240 disease related deaths occurred in 2016 [6]. This means at least 69% of ovarian cancer patients will succumb to their disease [7]. The disease is often misdiagnosed due to its elusive symptoms resembling other medical disorders [8, 9] and lack of reliable biomarkers for early detection [10], therefore, most patients would present with an advanced stage by the time the disease is discovered [11].

Only approximately 20% of ovarian cancers are diagnosed in stage I, which is characterised by cancerous cells forming a tumour that still lie within the ovary [12]. These patients have a chance of being cured with conventional surgical approaches and have survival rates of up to 90%. Unfortunately, the remaining 80% are diagnosed after the disease has progressed to stage II or higher [7]. As such, there are limited treatments for these patients and hence the recurrence of the disease and a low 5 year survival rate are typically observed [13, 14].

The first line of treatments, the debulking surgery and chemotherapy, are unlikely to cure the advanced disease and ultimately most of the patients have a poor outcome [11]. Due to these limitations a rationale for the development of effective targeted therapeutics is critical to improve the survival rates and quality of life of those patients'. Relative to other gynaecological cancers, advanced ovarian cancer has a poor 5 year survival rate of 36% compared to rates of 61% and 73% for cervical and endometrial cancers respectively [15]. According to a report from New Zealand Gynaecological Cancer Foundation the 5 year survival rate for ovarian cancer patients is 39% compared to 72% for cervical cancer, 78.5% for uterine cancer and 87% for breast cancer [16].

1.2 Cancer

All dividing somatic cells proliferate through the same highly conserved, basic cell cycle sequence [17]. Cells undergo the five distinct phases of the cell cycle: quiescence (G_0), Gap1 (G_1), DNA replication/synthesis (S), Gap2 (G_2) and mitosis (M) in order to produce new cells. Proteins and RNA synthesis as well as DNA repair occur in G_1 phase [18]. If these activities are prolonged then cells may go into quiescence and enter the G_0 phase (resting phase) [19]. Most cells would differentiate into the G_0 phase or re-enter the cycle following quiescence period [18, 19]. The next phase is the S phase were DNA synthesis occurs. At this point cells enter the G_2 phase with the newly replicated DNA in preparation for nuclear division and mitosis which occur during the M phase [20]. This synchronised transition through the different phases of cell cycle, which is facilitated by cyclins and cyclin dependent kinases [21], is pivotal for the maintenance of genome stability [18]. Normally cell proliferation is tightly controlled by different regulatory pathways, hence cell numbers are kept down via cell cycle check points e.g. pausing the cell cycle and/or promoting programmed cell death [17, 21].

Cancer cells undergo mutations that may lead to dysregulation of the cell cycle proteins and the checkpoint genes. This allows for a prolonged active phase of replication, dysfunctional apoptosis [18, 20], chromosome damages, aneuploidy (the presence of an abnormal number of chromosomes) and consequently malignant transformation. Genes that undergo mutations that lead to cancer are referred to as proto-oncogenes and typically encode growth and proliferative proteins and DNA repair proteins [17]. Cells with these mutations are prone to sustained proliferation and to evade cell death signals. Consequently, cells divide and proliferate in an uncontrolled manner forming tumours [18].

There are eight hallmarks that are suggested to be common to most types of cancers. Each hallmark offers a distinct role in the development, progression and recurrence of the tumour [22, 23]. These hallmarks are:

1- Sustaining proliferative signalling.

2- Evading growth suppressors.

3- Resisting cell death.

4- Enabling replicative immortality.

5- Inducing angiogenesis.

6- Activating invasion and metastasis.

7- De-regulating cellular energetics and metabolism.

8- Avoiding immune destruction.

The word tumour describes a broad range of solid or fluid filled cystic lesions that may or may not be related to cancer and the term "tumour" is usually used without a reference to the physical characteristics of size, shape, site, or type of the lesion [24]. Eighty percent of the ovarian tumours are benign tumours and mostly affect young women of 20-45 years of age. They usually increase in size but remain at their location and once removed they rarely appear again. Pre-malignant tumours are mostly found in older women; they tend to grow faster and might re-appear again after removal. Malignant tumours, however, are known to be life threatening and might re-appear locally and/or distally due to metastasis. Usually women with these tumours are aged 45 -65 years [25, 26].

Cancer is a term that refers to a broad group of diseases (there are more than 200 different types of cancer) that are characterised by mutations and unregulated cell growth. There are thought to be more than 60 organs in the human body where cancer can occur. A variety of factors e.g. hereditary (familial germline mutations in *BRCA1* and *BRCA2* genes) [27], environmental (chemicals, smoking), viral (Epstein-Barr virus, HPV) or bacterial infections (Helicobacter pylori), diet (low- roughage/high-fat, high nitrosamine intake), radiation (UV exposure, diagnostic exposure to CT scan, therapeutic radiation) , hormonal (exogenous oestrogen replacement therapy), inflammatory diseases (ulcerative colitis), and occupational exposure (dye and rubber manufacturing, asbestos mining, construction work, PVC manufacturing, petroleum industry) may contribute to the development of the disease [2, 15, 28].

As detailed above, in cancer the cells undergo mutations that involve the activation of proliferative signalling pathways triggered by numerous factors including autocrine stimulation (growth factors produced by the cancer cells) [29], downregulation of tumour suppressor genes, for example BRCA1 and BRCA2, the proteins that normally preserve intact chromosomal structure [30]. The factors described above cause the cells to divide and grow uncontrollably, to evade cell death signals and thus to form malignant tumours [22, 31]. The transformed cells may migrate from the original tumour via the bloodstream, the lymphatic system or through direct implantation [32] to invade surrounding tissues and other organs, a process that is referred to as metastasis [33].

1.3 Gynaecological Cancers

The term gynaecological cancer refers to malignancies of female genital tract, vulva, vagina, cervix, uterus and ovaries [34]. These will be described briefly below before a more in depth consideration of ovarian cancer.

1.3.1 Cancer of the Vulva

Cancer of the Vulva represents approximately 4% of all gynaecological malignancies and it is mostly diagnosed in early stages (I and II) of its development [35]. Therefore, it carries a good prognosis and a 5 year survival rate of greater than 75-90% for stages I and II. This then decreases to 54% for stage III and 16% for stage IV [35, 36]. An increasing incidence of cancer of the vulva is suggested to be due to the increased numbers of older women, mainly those infected with human immunodeficiency virus (HIV). HIV causes immune-T cell suppression, which may render the patient to be more susceptible to Human Papilloma Virus (HPV)-related cancers. [37].

1.3.2 Vaginal Cancer

Vaginal cancers are mostly secondary metastasis from nearby organs and primary carcinoma of the vagina is very rare with approximately only 0.45 cases per 100,000 women [6, 38]. Vaginal cancer incidence increases with increased age reaching its peak at around 80 years and is usually lower in Caucasian women in comparison to other ethnicities [39]. This cancer is also linked to HPV infections as according to a systematic review in 2009, 65% of the

patients with invasive vaginal cancer had HPV infections [40]. Other risk factors that contribute to vaginal cancer include multiple sexual partners, an early age of first intercourse and smoking, as well as women with history of vulvar and cervical cancer [41]. The disease has a 5 year survival rate of between 45-68% for the various stages [42, 43].

1.3.3 Cervical Cancer

Cervical cancer is the most widespread gynaecologic cancer worldwide in younger age groups of women. It ranks the 4th among all female cancers [44]. The most common predisposing factor for cervical cancer is HPV infection, other factors include multiple sexual partners, intercourse at teenage and grand multiparity [45]. Most disease related deaths reported in the USA were among women with low socioeconomic status [38]. Patients generally have a good prognosis if the disease is detected while still in stage I with no lymph node involvement (a 3 year survival rate of 86%), however, in the same stage, with positive lymph nodes the rate is lower (74%). Rates become even lower with increased disease stage and metastasis in the lymph nodes [45].

1.3.4 Endometrial Cancer

Endometrial cancer is a common gynaecological cancer and is the seventh leading cause of cancer related deaths among women [38]. There are several factors that contribute to the development of endometrial cancer of which obesity is the most common. The high levels of oestrogen and other obesity related hormones such as leptin, insulin and insulin-like growth factor (IGF) released from the adipose tissue may induce uncontrolled endometrial cell proliferation [46, 47]. Additionally, the prolonged use of exogenous oestrogen therapy for relieving the menopausal symptoms is also another etiological factor [48]. Other risk factors include early menarche, late menopause [49], low parity and age, where post-menopausal women over 55 years of age are more prone to develop endometrial cancer [50]. Diabetes,

hypertension [51, 52], smoking and family history [50] may also contribute to the development of endometrial cancer. Fortunately, post-menopausal bleeding is the most common symptom and often leads to early diagnosis and early treatment. Therefore women with endometrial cancer have a better prognosis than other types of gynaecologic cancers and patients without metastatic disease have 5 year survival rates of 74% - 91% [53].

1.3.5 Fallopian tube cancers

Fallopian tube cancer has been considered to be very rare, accounting for < 0.5% of all gynaecological cancers [54]. However, recent histologic, molecular and genetic studies suggest that about 40-60% of high grade serous carcinomas of the ovary may have been originated from tubal fimbria [30]. This means that tubal carcinoma may have been under estimated and, as is the case for ovarian cancer, the presentation of fallopian tube carcinomas resembles various medical disorders often leading to late diagnosis and poor prognosis[30].

1.3.6 The Ovaries and Ovarian cancer

1.3.6.1 Anatomy of the Ovaries:

The ovaries are two small organs within the female reproductive system; they have the shape of an almond and are located on both sides of the uterus [55]. The two organs are attached to their corresponding fallopian tube by the mesovarium. The latter is a membrane consisting of a double layer of peritoneum that supplies the ovaries with blood vessels, nerves and lymphatics. The ovaries are also attached to the lateral pelvic wall via infundibulo-pelvic ligaments (the thickened parts of the broad ligaments which suspend the ovaries to the lateral pelvic wall) [56]. The measurements of a normal ovary including longitudinal, transverse and antero-posterior diameters are 2.5-5cm, 0.7-1.5cm, and 1.5-3cm, respectively [55, 56].

The ovaries are formed of several types of cells from different origins. Histologically, the surface of the ovary is covered by a single layer of cuboidal coelomic epithelial cells [57]. The cortex consists of the main bulk of the ovary containing a large number of Graafian

follicles. The development of these follicles is a characteristic of the active sexual years [57]. The Graafian follicles are covered with spindle shaped theca cells and the cavity of each follicle contains the ova with its germ cells. The ova are surrounded by granulosa cells within a gonadal stroma [58]. Other contents of the ovarian stroma, following ovulation, include the corpus luteum, corpus albicans (both lined internally by luteinised granulosa cells), connective tissue cells and smooth muscle fibers [59-61]. Connective tissue fibers, smooth muscle cells, lymphatics, nerves and clusters of hilar Leydig cells make up the medulla of the ovary. This wide range of histologically distinct cell types and tissue varieties contributes to the disparate types of ovarian tumours and therefore there is a need for variable types of treatments that aim for particular targets.

The ovaries have two main roles. The first role is the production of the female hormones progesterone and estrogen and small amounts of testosterone [62, 63]. These hormones control the development of secondary sex characters of females e.g. they control the development of the breasts, body shape, and body hair. They also control the menstrual cycle and pregnancy. The second role of the ovaries is to store and produce ova [64]. A female ovary contains a huge number of minute ova (300,000-400,000) but only around 400-500 ova will mature and be released between menarche and menopause [63]. Their release is controlled by pituitary follicular stimulating hormone (FSH) and luteinizing hormone (LH) [65]. Every month when a female is at reproductive age, ovarian follicles start to grow in different stages, yet, only one or two reach the full size stage and will be released while the rest will disintegrate. The ovum travels to the uterus through the fallopian tube [66] reaching the endometrial cavity where if it remains unfertilised it will disintegrate as part of the sloughing off of the endometrium at the end of the menstrual cycle [63]. **Figure.1.1.** illustrates the diverse cellular biology of the ovary, fallopian tube and uterus [67].

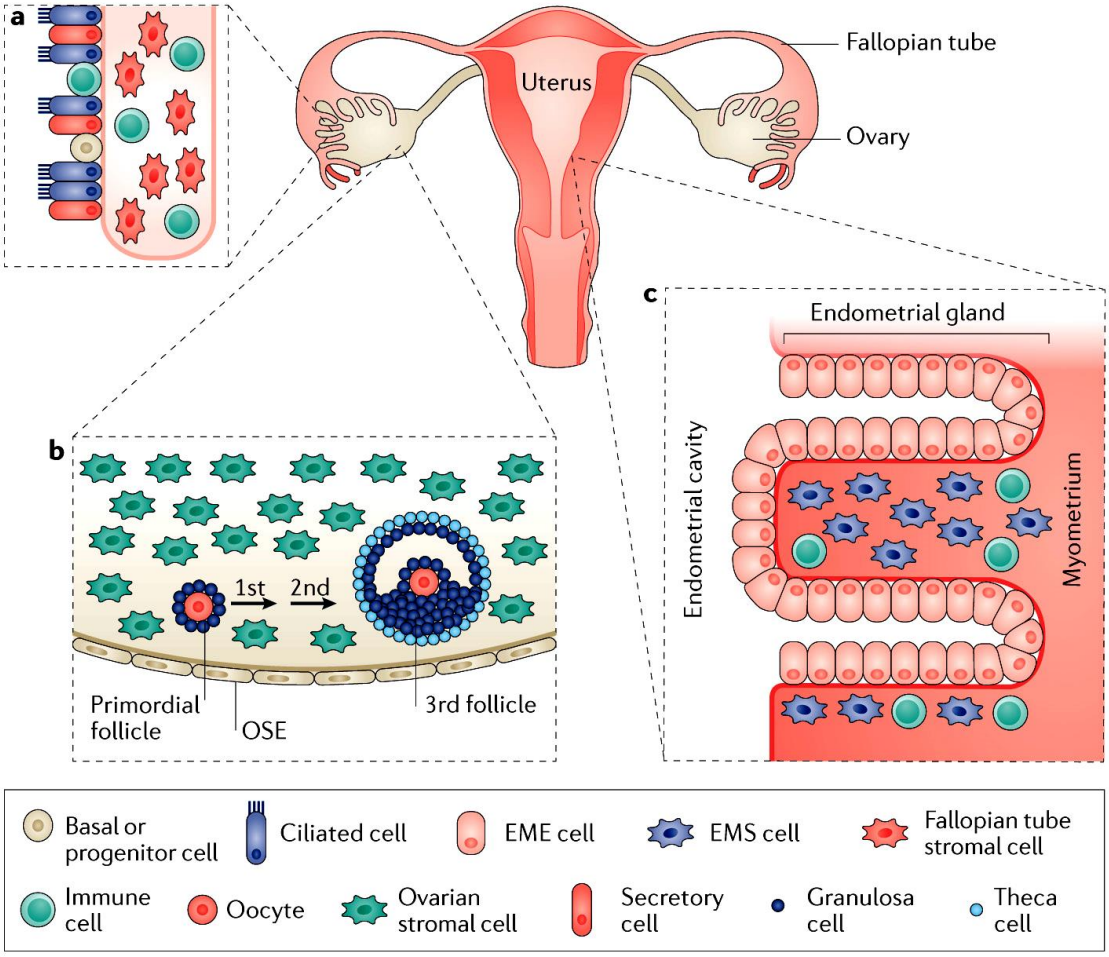


Figure.1.1. Anatomy and biology of the ovary, fallopian tube and uterus. The functional cells that are relevant to organ physiology and ovarian tumorigenesis. **a** | The secretory and ciliated cells in fallopian tube epithelium. Immune cells, which are present in the epithelium and stroma. **b** | Cells of the ovarian cortex, ovarian surface epithelium, stromal cells, and various stages of ovarian follicles. Granulosa cells and theca cells. **c** | The endometrial epithelium (EME) and endometrial stroma (EMS) [67]. Image permission was purchased from NATURE REVIEWS | CANCER.

1.3.6.2 Cancer of the ovary

Ovarian cancer is the leading cause of mortality in women with gynaecologic cancers [68]. In the past, ovarian cancer was considered to be one disease while it is now recognised as a group of diseases with distinct biological and histopathological appearances [69]. The rates of the disease vary in different regions of the world. Europe and the United States report the highest incidences of the disease followed by Australia and New Zealand, while Asia and Africa reported fewer incidences as shown in **Figure.1.2.** [70]. The variation in ovarian cancer incidence between different regions is suggested to be linked to smaller family sizes, high socioeconomic levels, high fat diets, older age and genetic background e.g. Caucasian descent.

Many pre-disposing factors have been suggested in the development of ovarian cancer. These include advanced age (women in the age range of 40-60 years are more susceptible than younger individuals) [59] and genetic mutations. 5-10% of ovarian cancer patients have been found to have a family history of ovarian, fallopian tube and peritoneal cancers as well as mutations including *BRCA1* and *BRCA2* [12, 71, 72]. Other factors include the environment, nulliparity [73, 74] and Caucasian descent. It has also been suggested that prolonged ovarian stimulation, for example through IVF, may increase the risk of developing ovarian cancer, however, this link is yet to be ascertained [75]. It should also be noted that pregnancy, breast feeding, and oral contraceptives may reduce the probability of ovarian cancer [76, 77].

Mutations in *BRCA1/2* are found in 5-15% of breast and ovarian cancers [78-80] and these have several copy number aberrations (CNAs) in high grade serous ovarian cancer (HGSOC). *TP53* mutations are also present in 96% of HGSOC according to the Cancer Genome Atlas [79]. *Tp53* encodes a protein that which terminates cell cycle progression, stimulates DNA repair, and activates apoptotic pathways [81, 82]. Thus cells with *TP53* mutations are more prone to retain and pass down damaged DNA, resulting in aggressive tumours. Epigenetic factors have also been implicated in the development of advanced ovarian cancer, for example alternative splicing of histone variants type H2A [83]. Overexpression of growth factor and /or adhesion receptors [84-86] have also been shown to alter the genome-wide methylation patterns in breast and ovarian cancers [87, 88].

It's commonly agreed that there are three cellular origins of ovarian cancer: the epithelium, germ cells, and stromal cells [89, 90].

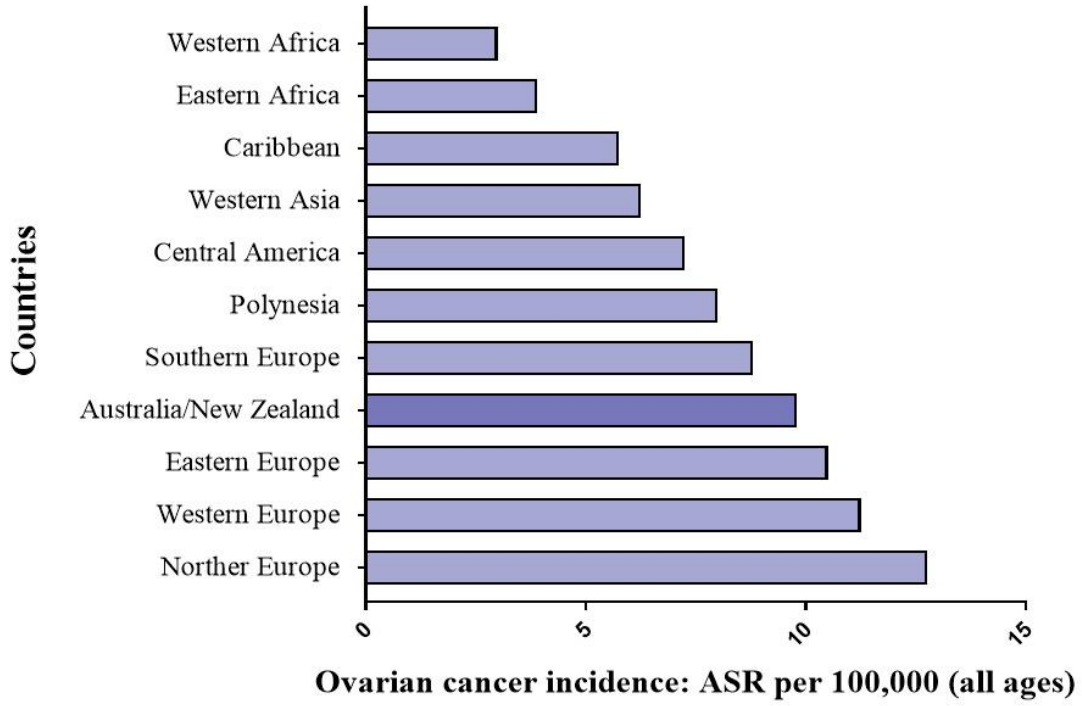


Figure.1.2. Age Standardised Incidence Rate (ASR) of ovarian cancer by region across the world. Image was taken with permission from IARC [70], and modified by the author to show the incidence of ovarian cancer primarily in New Zealand and Australia in comparison with selected worldwide populations.

1.3.6.3 Subtypes of Ovarian Cancer

The diverse cell types that form the ovaries mean that different types of ovarian cancer can have different histological and molecular properties [12] and the majority of tumour cells are often phenotypically distinct from normal ovarian cells [67]. The World Health Organisation (WHO) classified ovarian cancers according to tumour histology and cell origin into: epithelial, germ cell and sex cord stromal tumours [12, 91].

1.3.6.3.1 Epithelial ovarian cancer

Epithelial ovarian cancers are the leading cause of death from gynaecological cancers in USA and Europe [14, 76]. They account for 3% of all types of malignancies in females and are the second most common after uterine cancer [92]. Well established predisposing factors for acquiring epithelial ovarian cancer include family history (a first degree relative with the disease), early menarche, late menopause, old age and nulliparity [70]

Patients with familial susceptibility have a life time risk of acquiring epithelial ovarian cancer of 10-40% [12]. However, it has also been reported to occur sporadically at a rate of 1.7% (in a population of women without family history) [12]. This group of ovarian tumours are the most common types of ovarian cancers representing nearly 90% of all ovarian neoplasms (both benign and malignant) [92] and 98% of ovarian cancers [93].

Epithelial ovarian cancers originate from the mesothelial lining of the ovaries (which has the ability to differentiate into epithelial or mesenchymal tissue) either from the ovarian surface epithelium (OSE) or from its invagination into the ovarian cortex forming an ovarian cyst [94-97]. Based on the heterogeneity of different types of Müllerian epithelia and the histopathological, immunohistochemistry, and molecular genetic analysis [69], a recent guidelines report published in 2014 by the International Federation of Gynaecology and Obstetrics (FIGO), divided epithelial ovarian cancer into 5 subtypes: serous, mucinous, endometrioid, clear cell and undifferentiated carcinomas [69].

Although categorized as one group, these cancers are distinct entities differing in their molecular, biological, pathogenesis and clinical behavior [98]. According to their clinical behavior the tumours from each subtype can be further sub-categorised into: benign,

borderline, and malignant [59, 70, 98]. Images of the histological subtypes are shown in **Figure 1.3.** [99].

1.3.6.3.1.1 Ovarian Serous Carcinoma

Serous carcinoma is the most common type of epithelial cancer [91] accounting for 70% of all ovarian epithelial cancers [100] and is a major cause of death in patients with epithelial ovarian cancer. It originates from the Müllerian epithelium and is characterised by peritoneal dissemination [7, 101, 102]. Based on their nuclear atypia and mitosis, serous ovarian carcinomas can be classified into low grade and high grade carcinomas [103]. These differ in their genomic and molecular characteristics in that low grade serous carcinomas exhibit minimal nuclear atypia and low levels of mitosis ($\leq$ 12 per 10 high-power fields); while high grade carcinomas show significant nuclear atypia and mitosis [103]. Cytogenetic and single nucleotide polymorphism (SNP) analyses have revealed that low grade serous carcinomas exhibit fewer molecular abnormalities than high grade serous carcinomas [104, 105]. For example, low grade serous carcinoma have wild type *TP53* and mutations in either *KRAS, BRAF* or *ERBB2 (*HER-2*)* are common, while high grade serous carcinomas are characterised by *TP53* and *BRCA1/2* mutations and amplification in *CCNEI* (encoding cyclin E1) [92, 98]. The ErbB receptor (described in more detail below) has been shown to be significantly depleted in serous ovarian tumours. However, 5.1% were found to harbour the amplification reported by TCGA for high grade serous ovarian carcinoma [80].

Given that these two ovarian serous carcinomas present with different morphological features and arise from different genetic pathways [105-107], Singer and colleagues categorized them into type I and type II tumours [108]. While type I are low grade tumours that progress in a sequential fashion from "adenoma-borderline tumour-carcinoma", type II are high grade tumours that develop directly on the surface epithelium i.e. without precursor lesions [92], **Table 1.1** demonstrates some of the key differences between high and low grade serous ovarian carcinomas.

Table 1.1 Some of the key differences between high and low grade serous ovarian carcinoma (HGSOC and LGSOC).

HGSOC	**LGSOC**	Ref
Significant nuclear atypia	Minimal nuclear atypia	[103]
Significant mitosis	Low levels of mitosis (≤ 12 per 10 high-power fields)	[103]
High molecular abnormalities	Few molecular abnormalities	[104, 109]
Mutations in *TP53*	Wild type *TP53*	[92, 98]
Amplification in *CCNEI* (encoding cyclin E1)	Mutations in *KRAS*, *BRAF* and *HER-2*	[92, 98]
Type II tumours	Type I tumours	[108]

1.3.6.3.1.2 Molecular subtypes of HGSOC

HGSOC is of particular interest due to its high incidence of disease related deaths and low overall survival rate [79]. Using high-density expression oligonucleotide microarrays to profile 285 well-annotated serous and endometrioid invasive ovarian, fallopian tube, and peritoneal cancers, Tothill and colleagues [110] were able to identify six distinct molecular subtypes of HGSOC.

1- C1 High stromal response Tumours

2- C2, High immune signature Tumours

3- C4, Low stromal response tumours.

4- C5, Mesychymal low immune signature tumours.

5- C3, Serous low malignant potential (LMP) tumours

6- Low grade early stage endometrioid tumours.

[110].

A follow up paper by Leong and colleagues [100] in 2015 suggested further description to these molecular subtypes where C1 were suggested to show intensive dysmoplasia, an epithelial–mesenchymal gene expression signature, and linked with primary treatment failure and poor survival. Immune reactive C2 tumours were characterised by extensive intra-tumoural T-cell infiltration and generally have a better prognosis. However, C4 demostrates an intermediate outcome and the gene expression signature of these tumours shares some features with serous borderline tumours. Finally, C5 proliferative tumours show low expression of differentiation markers such as CA125 and limited infiltration with poor outcome [100]

Diversity in clinical responses is also recognized between the low and high serous ovarian carcinomas. The initial response of high-grade serous carcinoma to standard chemotherapy of platinum and taxanes (Carboplatin and Paclitaxel) is good following debulking surgery. However, 20-30% of patients experience relapses of the disease within less than 6 months

[111]. This relapse appears to be associated with the nature of mutations observed in the high grade serous carcinoma [112], specifically amplification of *CCNE1* which have been shown to be a poor prognostic factor [113]. A five-year survival rate of 20-35% is expected for patients with serous ovarian carcinoma [12, 114].

1.3.6.3.1.3 Endometrioid cancer

These tumours are common mostly in women in the age range of 50-59 years. They account for 10-20% of all epithelial ovarian cancer cases [92]. A 5-year survival rate of 40-63% and good prognosis is observed in ovarian endometrioid carcinoma [9, 114, 115]. This type of epithelial ovarian cancer can also be classified into low grade endometrioid and high grade endometrioid.

At the molecular level, low grade endometrioid ovarian cancer is typically a type I tumour molecular subtype which is genetically stable and found to carry several somatic mutation such as *PIK3CA, PTEN, KRAS, BRAF*, and *CTNNB1* (the gene encoding for β-catenin protein). The β-catenin protein was found to be involved in cell-cell adhesion and gene transcription processes and seems to play a major role in the development of endometrioid ovarian carcinoma [92, 116]. High grade endometrioid ovarian carcinoma is classified as a type II tumour, which is aggressive, unstable with > 95% of cases carrying *TP53* mutations [117].

1.3.6.3.1.4 Mucinous adenocarcinoma

Mucinous ovarian carcinoma accounts for 5-20 % of epithelial ovarian cancers and patients with advanced stages have worse prognosis and survival rates [118]; the 5 year survival rate is 40-69% for patients with mucinous ovarian cancer. They are heterogeneous tumours in terms of cellular components as well as the degree of differentiation [92]. Mucinous ovarian tumours have been found to retain a mixture of benign, borderline and malignant cells in the same neoplasm suggesting a step by step progression. Mucinous ovarian carcinomas were found to carry mutations in 17q12, which encodes for *ERBB2* (HER-2) and the gene was significantly amplified [119]. The presence of *KRAS* mutations in all three areas (benign, borderline and malignant) suggests its involvement in the early stages of the development of

mucinous ovarian carcinoma [92, 120, 121]. RAS oncogene is a small GTPase within the intracellular proliferative pathways that leads to mitosis and thus mutations in this gene was found to enhance cell proliferation [122, 123]. ECM proteins including E-cadherin and matrix metallo protein (MMP), which are cell adhesion and migration molecules, are also found expressed on mucinous ovarian cancer cells [124, 125].

1.3.6.3.1.5 Clear cell ovarian carcinoma

Clear cell ovarian carcinomas represent between 3-10% of epithelial tumours. The 5 year survival rate is 35-50 % in patients with clear cell carcinoma [9, 12, 114, 126]. A genome wide analysis revealed molecular genetic somatic inactivation in *ARID1A* genes [127, 128]. Studies of morphologic and molecular characteristics of clear cell ovarian carcinoma have revealed a sequential pattern of progression [127, 129]. This type is characterised by activating mutations in *PI3KCA* and amplification of chr20q13.2 (encodes for *ZNF217* oncogene) [127, 130].

Regardless of the stage upon diagnosis, the response rate to standard platinum-taxane chemotherapy is very low and the disease has a significantly poor clinical outcome [131-133]. Clear cell ovarian carcinomas have found to carry a unique genetic profile in that they have upregulation of certain genes including *HNF-1β* [134, 135]. *ARID1A* and *PIK3CA* mutations are more frequent (>40%) in clear cell carcinoma compared to other histological types of epithelial ovarian cancers [127, 128, 136, 137].

1.3.6.3.1.6 Undifferentiated ovarian carcinoma

Undifferentiated ovarian carcinoma is a rare type of ovarian tumour that usually present as large, solid, necrotic tumours. According to the WHO, these tumours are characterised by cells that show no differentiation or rare areas of differentiation [115]. It accounts for just 1% of all epithelial ovarian cancers [9, 12] and in most cases the patients would present with metastatic tumours and therefore a poor prognosis is often observed [138].

The 5-year survival rate is approximately 11-29% [12, 114]. According to its molecular and clinical properties, undifferentiated ovarian carcinoma is suggested to be a type II tumour [97]. Like all other cancers with type II molecular profiles, this ovarian carcinoma exhibits morphologic and molecular homogeneity with an unstable tumour that primarily harbours *TP53* mutations [97].

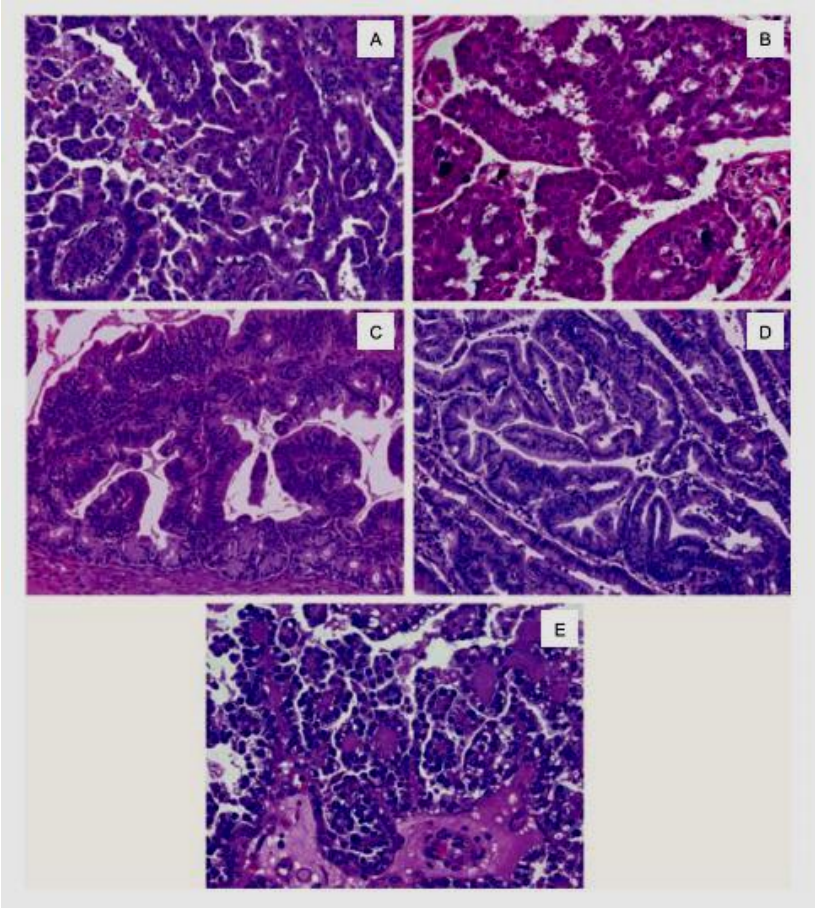


Figure 1.3. Histopathological images representing the different subtypes of epithelial ovarian carcinoma which account for 98% of all ovarian malignancies. A) High grade serous carcinoma. B) Low grade serous carcinoma. C) Endometrioid carcinoma. D) Mucinous carcinoma. E) Clear cell carcinoma. Image taken from World Cancer Report 2014 [99] with permission from the author Dr Jamie Prat [69].

1.3.6.3.2 Ovarian Germ cell tumours

Ovarian germ cell tumours are a group of tumours that originate from primordial germ cells derived from the embryonal gonads [139]. These types of cancers represent 3-7% of ovarian cancers, however most tumours that originate from germ cells of the ovary are benign [25, 60, 69, 140]. They can be classified as dysgerminoma, yolk sac tumour, embryonal carcinoma, poly embryoma, nongestational chorio carcinoma, mixed germ cell tumours and teratoma [141]. Ovarian malignant mixed germ cell tumours are highly aggressive and usually affect young women below the age of 20 years. However, if diagnosed at an early stage, a good prognosis is expected and the patient's fertility could be spared [141, 142].

1.3.6.3.3 Ovarian Sex cord-stromal tumours

Ovarian sex cord-stromal tumours originate from the sex cord and mesenchyme of the embryonic gonads. Most of these tumours are benign or are of low grade malignancy with a good prognosis and usually affect younger women <40 years of age. Hormone secreting sex-cord tumours represent 90% of functioning ovarian tumours [143]. Granulosa cell (estrogenic), and androblastoma (androgenic) are examples of sex cord tumours, which are generally associated with symptoms of hormonal effects. Ovarian sex cord tumours represent 7% of all cancerous ovarian tumours [60].

1.3.6.4 Stages of Ovarian Cancer

According to FIGO staging ovarian cancers can be categorised into four stages and each of these can be split into three subdivisions. These are summarised in **Table.1.2**.[12, 71, 144, 145]. Patients with stage IA tumours in which the disease is restricted to one ovary, and there is no ascitic fluid have a good prognosis and this can be treated by surgery. Stage IB tumours can be in both ovaries but without malignant ascites. Following surgery alone, there is a 5-year survival rate of 90 % for both IA and IB [146].

Stage IC is characterised by rupture of the tumour surface, malignant ascites and can be limited to one or both ovaries. Stage II ovarian cancer presents with pelvic extension and can be further classified into stage IIA in which tumours may be present in the uterus or fallopian tube and stage IIB, in which the tumour reaches other pelvic organs (bladder, rectum or pelvic side walls). Stage IIC is identified by tumour extension to other ovary, with a ruptured surface

and malignant cells in the ascites. The 5 year survival rate for stage IC and stage II reaches 80% when postoperative adjuvant therapy is given [12, 147, 148].

Stage III of the disease involves spread to the lymph nodes or upper abdomen and is further sub divided into stage IIIA in which the disease is found in microscopic small deposits in the omentum. Stage IIIB deposits are 2 cm or less and are outside the pelvis. Stage IIIC of the disease has gross deposits more than 2 cm and these are outside the pelvis. Stage IV of the disease involves the dissemination of the disease to distant organs such as pleural space, the liver and /or splenic parenchyma. For patients with advanced stages of epithelial ovarian cancer (stage III and IV), following debulking surgery with residual disease of 1 cm or less, the 5 year survival rate is around 20-30 % and may reach 10% if insufficient debulking is achieved [12, 147-149].

The prognosis for patients with ovarian cancer can be complex and depends on several factors that contribute to the development of the disease and the outcome of response to management. These factors include the stage of the disease, histopathological findings, size of the tumour mass, the amount of remnants of tumour after surgery, and the presence of malignant ascites.

Table.1.2. FIGO Staging system for epithelial ovarian cancer. the stages of ovarian cancers as described by FIGO in accordance to the level of advancing and clinical outcome [12].

Tumour stage	Description
Stage I	Tumour only involving the ovaries
IA	Only one ovary is affected without ascites
IB	Both ovaries involved without malignant ascites
IC	One or both ovaries with ascites.
Stage II	One or both ovaries with local metastasis.
IIA	The tumour is spread to the uterus.
IIB	Metastasis reaches other pelvic organs, such as bladder, rectum, and pelvic side walls.
IIC	One or both ovaries and the tumour spread to the uterus and other pelvic organs causing ascites formation.
Stage III	Tumour affecting one or both ovaries with extensive spread of the disease to the peritoneal cavity and pelvic lymph nodes.
IIIA	Minute spread of the disease towards upper abdomen reaching the omentum.
IIIB	Large metastasis that can be recognized by naked eye, over 2cm in size.
IIIC	In addition to findings in 3B, metastasis to lymph nodes occurs.
Stage IV	Further metastasis reaching distant organs, i.e., liver, spleen, and plural cavity

1.4 Intraperitoneal dissemination of Ovarian Cancer

Whilst the primary tumour is generally confined to the ovary, the cells may be able to become mobile and spread as they undergo epithelial- mesenchymal transition (EMT) to attain motility. EMT is a crucial progressive mechanism that allows polarised epithelial cells to transform into motile mesenchymal cells [150, 151], which enables the exfoliation of cancer cells from the primary tumour and their migration to local and distant organs [151-153].

This transition involves changes in the behaviour of molecules that are involved in adhesions [33, 101, 154, 155]. Malignant cells that disseminate from the primary tumour survive as single cells or aggregates floating in the excess body fluid that can accumulate (ascitic fluid) as a result of ascites [156]. It has been suggested that intraperitoneal fluid and peritoneal surface motion (peristalsis) is a key driving in cell migration and further spreading through the abdominal cavity [157-159]. The seeding of malignant cells into the peritoneal cavity and the subsequent metastasis are often associated with the formation of the malignant ascites which is a manifestation of advanced stages of the disease. This is also the case for several other types of cancers but occurs most prominently in ovarian cancer [153, 160].

Once cells reach the mesothelial lining of the abdominal cavity, they lose their migratory capacity undergoing mesenchymal epithelial transition (MET), which is the reverse process of EMT. This transition is believed to be a driver of cells seeding on the peritoneal cavity and other organs, thereby seeding a new secondary tumour as the cells regain their epithelial characteristics such as polarity and the ability to form cell junctions [154]. **Figure 1.4** depicts a model for the intraperitoneal dissemination of advanced ovarian cancer cells [153].

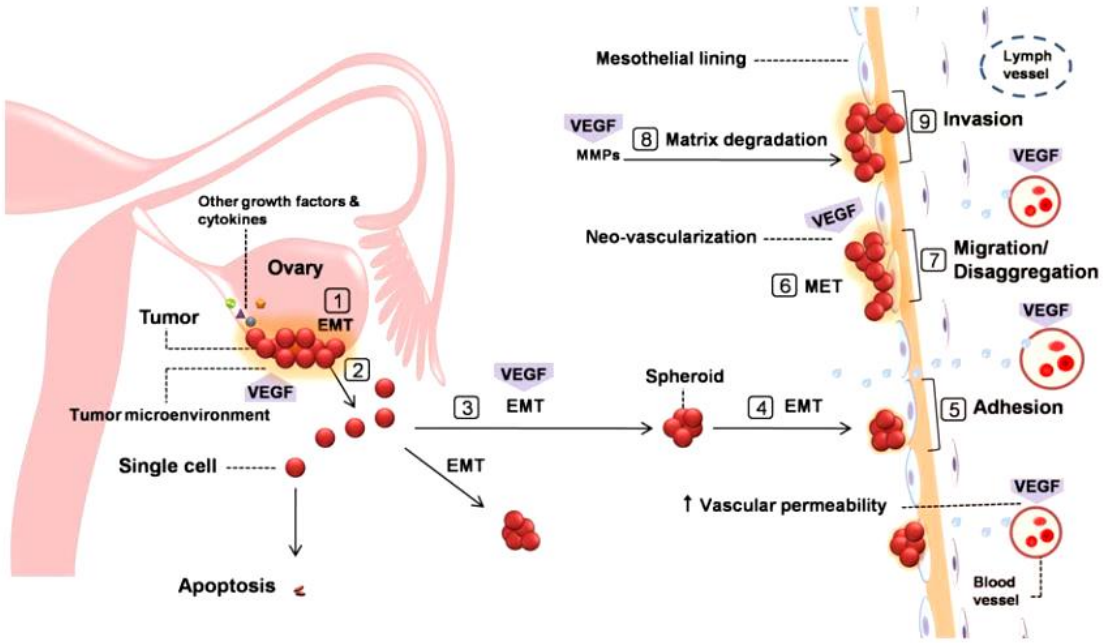


Figure 1.4. A proposed model for intraperitoneal dissemination of ovarian cancer. Primary tumours are generally confined to the ovaries. To metastasize, tumour cells undergo epithelial–mesenchymal transition (EMT) to attain motility and shed from the tumour, surviving as single cell or compact aggregates. Aggregates undergo invasive mesenchymal changes to maintain survival and motility. Upon reaching the abdominal wall cells adhere and implant on the peritoneum and mesothelial linings of pelvic and abdominal organs. To initiate metastasis cells undergo a reverse transition (MET). Through the activity of MMPs, matrix degradation occurs & and invasive tumour cells infiltrate the mesothelial lining and the extracellular matrix. Permission to use the image was granted from the authors [153].

1.5 Ascites: Malignant Ascites in ovarian cancer

Around 150-200 mL of free fluid is normally present in the pelvic cavity, especially at times of ovulation, and this is usually asymptomatic. Higher volumes (>1L) of ascitic fluid in the abdominal cavity can be a sign of several diseases such as heart failure, renal failure, liver cirrhosis, tuberculous peritonitis, and various cancers [161-165]. It has been reported that 10 % of cases presenting with ascites are due to cancer [164].

In the case of cancer, ascitic fluid can be tested for the presence of malignant cells by sampling via peritoneocentesis (aspiration of the fluid) [166] although definitive diagnosis requires cytological analysis of the fluid [164]. Ascitic fluid from diseases other than cancer would appear different, cloudy in the case of infections, have a clear straw- or light green colour in the case of liver cirrhosis, appear milky white in the case of lymphatic obstruction. In the case of malignant ascites, they would usually be light yellow colour with less or no sign of red blood cells apparent.

Malignant ascites can be defined as the accumulation of ascitic fluid containing cancer cells in the abdominal cavity [156], which can occur as a result of multiple factors such as lymphatic obstruction, increased vascular permeability and stromal immune cells associated with tumour transudate secretions [101, 156]. However, ascites can occur with both benign and malignant tumours [59]. The presence of cancer cells is the main feature of malignant ascites where the tumour sheds malignant cells into the ascitic fluid [164]. These cells form clusters and compact aggregates in order to survive. Ascites due to ovarian cancer account for 37% of malignant ascites, followed by pancreato-biliary cancer (21%), gastric (18%), oesophageal (4%), colorectal (4%) and breast cancer (3%) [167].

The components of malignant ascites can be divided into two main categories, cellular and acellular. Cellular components include malignant cells (single and aggregates), endothelial cells, adipocytes, and inflammatory cells. Acellular (soluble) components include growth factors (e.g. EGF, HGF and VEGF), cytokines, chemokines, ECM fragments and various proteins [168, 169]. These proteins include human serum albumin (HSA), which is usually found at high levels in malignant ascites due to increased vascular permeability [170]. HSA is a 585 amino acid, single chain protein carrying several negative charges that can bind

reversibly to many substances including drugs and hence plays a pivotal role in drug delivery [171-174]. This binding may cause significant pharmacodynamics alterations due to changes in drug properties including absorption, distribution, metabolism and excretion [175].

Additionally, the high levels of vascular endothelial growth factor (VEGF) from ovarian, colon, and gastric cancer cells may contribute to the formation of ascites. It has been suggested that the presence of VEGF in ovarian cancer is crucial for ascites formation [164]. The production of VEGF by ovarian cancer cells is suggested to be driven by several factors including growth factors [176]. Therefore, inhibition of VEGF dramatically halts the production of ascitic fluid [177].

1.6 Treatment of ovarian cancer

1.6.1 Surgery

Surgical procedures are the cornerstone treatment for tumours in the early stages of the disease. The aim of surgery is the excision of the entire tumour growth and is performed in patients with stage I/II ovarian cancers. In some early stages of ovarian cancer a good prognosis would be expected after surgery. However, since nearly two-thirds of ovarian cancer patients present with advanced stages of the disease by the time they are diagnosed debulking surgery is then recommended. Unfortunately, some of the tumour tissue may remain in the abdominal cavity after surgery[178]. Additionally, the advanced stage of the disease may lead to a deterioration of the patients' health and thus may affect the fitness of the patient for surgery. Another option for such patients is a reduction of the bulk of the tumour by chemotherapy prior to surgery [179].

1.6.2 Chemotherapy

Chemotherapy is the normal treatment for advanced ovarian cancer patients. Cytotoxic agents are used to target fast dividing cells. The rationale is that these agents slow down the tumour growth and induce cell death. Newly diagnosed ovarian cancer patients have high response rates to such platinum-based chemotherapy. Therefore, primary chemotherapy has emerged as a preferable treatment prior to debulking surgery [68]. First line chemotherapy for advanced ovarian cancer patients is currently the therapeutic backbone which involves a

platinum agent (mostly carboplatin) and a taxane (mainly Paclitaxel) [180, 181]. Second line chemotherapy is an option when the patient is not responding to first line treatments. These include doxorubicin, etoposide, gemcitabine, ifosfamide and cyclophosphamide [182]. The resistance to chemotherapy is mainly characterised by a reduction in the efficacy of the drug to inhibit tumour growth over time. Recurrence of the disease is observed in 80% of cases with high grade serous ovarian carcinoma [181, 183]. Some suggested mechanisms for chemo resistance include 1) overexpression of multidrug resistance genes (MDR), which are basically responsible for pumping the drug out of the cells. 2) Alterations in drug metabolising enzymes (e.g. glutathione transferases). 3) Increased DNA repair and evading apoptosis due to mutations in tumour suppressor genes (e.g. *P53, BRCA1/2*). 4) Defective cell cycle checkpoints at the mitotic spindle step, causing to resistance to microtubule inhibitors [184].

A phase II trial of Bevacizumab, a humanised anti-VEGF monoclonal antibody, was conducted on patients with recurrent epithelial ovarian cancer by burger and colleagues [185]. The study included 62 patients with average age of 57 years. Two patients showed complete response while 11 showed partial (total response rate of 21%), 32 patients (51.6%) had stable disease and 40.3% had progression-free for at least 6 months [185].

Carboplatin is an FDA approved, platinum-based chemotherapeutic drug for the treatment of advanced epithelial ovarian cancer [186]. Carboplatin and cisplatin act by crosslinking interstrand DNA covalently binding with the DNA [186]. Paclitaxel is a member of the taxane mitotic inhibitor-based chemotherapeutic agents that have been approved as an effective treatment (alone or in combination with other chemotherapeutic agents) for several types of cancers including breast [187], prostate [188], bladder [189], NSCLC [190] as well as ovarian cancer [147].

Several drug trials have been attempted for the treatment of mucinous ovarian cancer but the lack of sufficient number of participants has been an obstacle. For example, in a trial conducted by McGuire and colleagues on treatment with cisplatin and cyclophosphamide vs. treatment with cisplatin and paclitaxel the total number of patients was 410 but only 14 of these had mucinous carcinoma [148]. Another trial with 680 supposed patients, for treatment with cisplatin and cyclophosphamide vs. treatment with cisplatin and paclitaxel, only 30

patients had this tumour [191]. However, a study by Shimada and colleagues [192], on 24 patients with mucinous ovarian cancer showed a response rate of 12.5 % when treated with platinum-based regimens [192]. The same study was also conducted on 189 patients with serous ovarian carcinoma showed a response rate of 67.7 %. This suggests that mucinous tumours may be platinum resistant.

The chemotherapeutic agents described above exert their effect by impairing mitosis, hence killing rapidly dividing cancer cells. However, in addition to malignant cells, there are normal cells that are frequently going through rapid division for example bone marrow cells, hair follicles, and gastro-intestinal lining epithelial cells [193]. Thus the drugs will target non-malignant cells as well as the cancer cells. This may cause numerous adverse effects that include mouth ulceration, nausea, vomiting, diarrhoea, alopecia (hair loss). Laboratory findings include low white blood cell count (neutropenia), myelosuppression (reduced RBCs production), and low platelets count (thrombocytopenia) [15]. The efficiency and/or toxicity of the chemotherapeutic drugs can be affected by several factors including the levels of drug absorption, distribution, metabolism, excretion and drug resistance [186, 194]. There is therefore a need for novel drugs that target only the malignant cells and which leave the normal cells unharmed.

1.6.3 Radiotherapy

Radiotherapy is considered one of the conventional methods for the treatment of cancer. The aim is to damage the DNA of the cancer cells at certain check points of the cell cycle [195], using radiation released from the decomposition of radioactive isotopes, or a radiation beam delivered by X-ray. Radiation dose depends on tumour type and volume to achieve the best cure with least toxicity. During the treatment, patients may experience nausea and fatigue [196].

Adverse effects of radiotherapy affect normal rapidly growing cells of the bladder (cystitis), rectum (proctitis), lung fibrosis and skin usually appear 2-3 months after completing the treatment. The skin is the first site where the radiation contacts the body and this can lead to the development of erythema due to inflammation of the skin and subcutaneous tissue [196].

For several decades, radiotherapy was the best treatment for primary ovarian cancer, but as chemotherapy advanced, trials to compare radiotherapy and chemotherapy proved superiority of the latter approach [196].

Pelvic radiotherapy for early stage ovarian cancer reduced the relapse rate in the pelvis yet showed no overall increase in survival rate [197]. Currently radiation is rarely used as first line therapy for ovarian cancer treatment. It is possible that combining radiotherapy and chemotherapy can improve disease control in patients with stage I or those with chemo resistance. Radiation can be achieved by external Deep X-ray therapy to one side of the pelvis, protecting the epsilateral hemi pelvis and thus protecting the other ovary and head of the femur. The other method is intraperitoneal instillation with radiocolloid agents which had been previously used for the treatment of patients with stage I ovarian cancer. However, it was did not decrease rates of relapse or improve survival rates [198].

Generally, this type of treatment is considered out of date because of the small number of patients that may benefit from it. Additionally, the patients are showing better tolerance to the new chemotherapeutic agents [199].

1.6.4 Hormone therapies

Several hormonal agents have been investigated in the treatment of advanced ovarian cancer. Studies on anti-estrogenic and aromatase inhibitors have been done on patients with recurrent ovarian cancer that show resistance to platinum group chemotherapy [200]. Aromatase inhibitors lead to short-term stabilisation of the recurrent disease in 50% of heavily treated patients [201, 202]. This was aimed at patients who have recurrent elevated tumour markers following initial remission of stage III and IV ovarian epithelial cancer [203]. Preclinical studies have shown that agents such as tamoxifen can inhibit the growth of ovarian cancer cells *in vitro* which provided a base for further investigation [204]. Hormones or hormone blocking agents may be used for the treatment of some ovarian cancers including ovarian stromal tumours. Oestrogen stimulates cancer cell growth through oestrogen receptors (ER) which are overexpressed in 36% of ovarian cancers [205].

Some available hormone therapies were approved by the FDA for the treatment of several cancers; these include drugs such as luteinising-hormone-releasing hormone agonist (LHRH),

also known as gonadotropin releasing hormone agonists (GnRH). The function of this group of drugs is reducing oestrogen release from the ovaries in pre-menopausal women. Several adverse effects were seen on patients including menopausal symptoms such as hot flushes, a dry vagina and osteoporosis. The latter occurs with prolonged administration. Another example is Tamoxifen; this treatment is mainly used for breast cancer and ovarian stromal tumours but rarely for advanced epithelial ovarian cancer. Tamoxifen is an anti-oestrogen agent that inhibits the stimulatory effects of circulating oestrogen. It shows similar side effects as the GnRH agonists except for the osteoporosis. However, it may increase the risk of serious deep vein thrombosis. Aromatase inhibitors including Letrozole (Femara), Anastrozole (Arimidex) and Exemestane (Aromasine) elicit a good anticancer response and have stabilised recurrent disease in patients with positive ER tumours. They function by blocking the enzyme aromatase which converts other hormones to oestrogen in post-menopausal women. These have been mainly used in breast cancer as well as relapsing ovarian stromal tumours following initial treatments (e.g. surgery and chemotherapy) [202, 206]. Adverse effects from the use of aromatase inhibitors include hot flushes, nausea and skeletal pain (muscles and joints), osteoporosis and bone fracture in prolonged use of treatments [203]. Hormone therapies are sometimes considered to be part of targeted therapies.

1.6.5 Immunotherapy

Immunotherapy involves the modulation of the immune system and is based on the theory that the immune system can recognise and destroy malignant cells as foreign bodies. New research is suggesting that it may be possible to overcome chemotherapy resistance in ovarian cancer by combining immunotherapy with chemotherapy [207]. There are two types of immune cells that have been investigated, fibroblasts (the stroma) and immune T cells. Fibroblasts lead to cisplatin resistance by releasing two components: glutathione and sesteine. Adding immune T cells to the fibroblasts can lead to disintegration of tumours. The T cells alter the metabolism of glutathione and sesteine in the fibroblast [208].

Trials of vaccinations of ovarian cancer patients with a variety of antigens including MUC-1 carbohydrate epitope (P53 peptide) and HER-2 peptide have emerged as promising methods of treatment [70]. Clinical trials using whole tumour cells as vaccine means immunising

against many antigens at the same time, however, no promising results have as yet been achieved with this method [209].

The *in vitro* generated tumour reactive T cells are used to test for the ability to identify intact cancer cell antigen found on cancer cell surface. New methods of treatment of cancer have been investigated, such as immunisation of patients with variety of immunogens. Because of delayed response to immunotherapy (tumour regression) there is confusion over the efficacy of cancer immunotherapy. This may be due to the use of inappropriate animal models in studies [210, 211].

1.6.6 Targeted Therapy

In the past decade, targeted therapy has emerged as a novel anti-cancer treatment that aims to inhibit specific proteins involved in carcinogenesis, tumour growth and angiogenesis The goal is for these treatments to be more effective than current treatments and to be less harmful to normal cells and thus cause less systemic toxicity [212]. The chosen targets are usually over expressed in cancer cells compared to low or no expression in normal cells, therefore the drugs have minimal effect on normal cells. Examples are the variety of targeted anti-cancer agents that are designed to block EGFR and HER-2. These include monoclonal antibodies (trastuzumab and cetuximab) and small synthetic inhibitors (gefitinib, erlotinib, lapatinib, canertinib, afatinib and dacomitinib) [213-223].

One of the most studied cell signalling pathways is the PI3K/Akt pathway, which has been shown to be important in many cellular processes [224], and found to be deregulated in many cancer cells including those in the ovary [106, 225]. Numerous PI3K inhibitors have been investigated as potential agents of targeted therapy and these have shown anti-tumour activities as single agents, or when used in combination with chemotherapy, in both *in vivo* and *in vitro* ovarian cancer models [226, 227]. The inhibitors of PI3K have shown good outcome in both PI3K mutant and wild type tumours in several cancers including breast and colorectal cancers [228, 229]. For example an orally available pan PI3K inhibitor, BKM120

was shown to have anti-tumour effects in a phase I/II clinical trials in several xenograft models of advanced solid tumours [230-232].

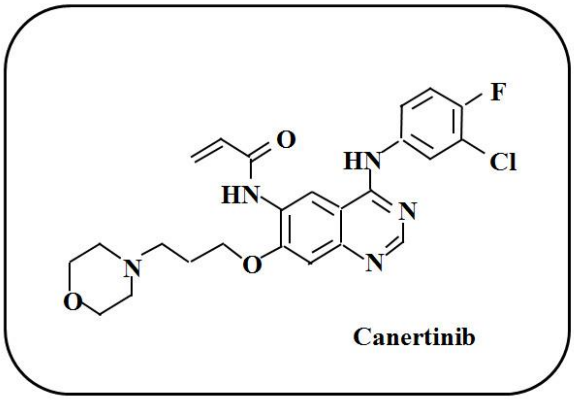
Poly (ADP-ribose) polymerases (PARPs) are a group of catalytic molecules involved in in numerous cellular activities including cell cycle progression, DNA repair and genomic stability [233]. PARP inhibitors have been shown to induce anti-tumorigenic activities in *BRCA-1* and *BRCA-2* mutant breast and ovarian tumours [234, 235]. A response rate of 30-45% to PARP inhibitors such as Olaparib was observed in platinum sensitive relapse cases of high grade serous carcinoma carrying mutations in *BRCA1/2* [236, 237].

Olaparib is an FDA approved targeted therapy for advanced ovarian cancer patients with *BRCA1/2* mutations [238]. The poor response from a fraction of ovarian cancer patients with *BRCA1/2* led Wang and colleagues to suggest combining PI3K with PARP inhibitors in ovarian cancer cell lines that have mutant or wild type *PI3K* [239]. In another study, the same authors demonstrated the effectiveness of this combination in ovarian cancer cell lines with wild type PI3K and suggested that concomitant downregulation of BRCA expression may probably be a biomarker for a patient's response to combined inhibition of PI3K and PARP in ovarian cancer [240].

Certain small synthetic molecules have been used as targeted drugs in clinical trials and this class of compound is of interest in the research described in this thesis. There are many small molecule inhibitors that have been approved by the FDA, these have been shown to be efficient in inhibiting the activity of the receptors in patients with breast, endometrial, colon and non-small cell lung cancer [241, 242]. These inhibitors target EGFR and HER-2 and include gefitinib and erlotinib, which are first generation reversible inhibitors of EGFR. These block EGFR activity, phosphorylation and downstream signalling molecules [243]. Afatinib and dacomitinib are second generation small molecule inhibitors that block EGFR and HER-2 by forming a covalent bond at the ATP binding site. These second generation inhibitors are irreversible, inhibit multiple EGFR family receptors and have high affinity for the binding sites. Therefore, they are suggested to be more efficient than first generation inhibitors [221, 244].

The first generation small molecule tyrosine kinase inhibitor canertinib (**Figure 1.5.** [245]), is an irreversible, pan EGFR inhibitor that permanently binds intracellularly to the ATP binding site of all 4 members of EGFR receptor tyrosine kinase family [245-247]. These two characteristics of canertinib (being a pan inhibitor and irreversible) increase the efficacy of the drug and these ensure prolonged availability in the system in addition to meaning that lower doses of the drug are effective [218]. Canertinib has shown anti-tumour activity in both *in vitro* and *in vivo* studies [248, 249] and has also shown dose-dependent inhibition of tumour growth in murine models [250, 251]. It has shown anti-tumour activity in both *in vitro* and *in vivo* animal models of breast cancer [248], and phase II studies with NSCLC and breast cancer are ongoing. However, in trials with ovarian cancer canertinib has exhibited limited efficacy when used as a single agent [219].

PHA665752 is a second generation small molecule reversible c-MET receptor tyrosine kinase inhibitor (with the structure shown in **Figure.1.6).** It inhibits ATP binding to the tyrosine kinase domain of the receptor, down regulating its phosphorylation and thus impairing cell growth, proliferation and migration, leading to apoptosis in *in vitro* cell culture models of oesophageal, gastric and NSCLC cancers [252-254]. Several *in vivo* studies have shown that PHA665752 inhibits c-MET phosphorylation and cell growth of tumour xenografts in athymic mice [255, 256]. Recently, PHA665752 was also suggested to work in combination with cisplatin and appears to reverse resistance to cisplatin in SKOV-3, OVCAR-3 and OV-90 ovarian cancer cell lines [257]. Furthermore, Jia and colleagues [258] demonstrated that a combination of PHA665752 with cetuximab inhibited *in vitro* and *in vivo* growth of human colorectal cancer cells [258].



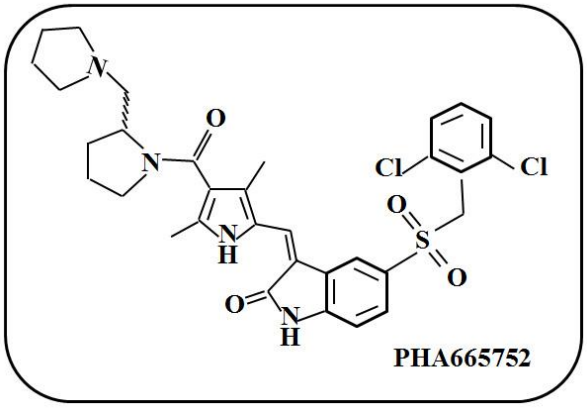


Figure 1.5. Chemical structure of canertinib **Figure 1.6.** Chemical structure of PHA665752

There have been numerous reports of clinical trials using a combination of small molecule tyrosine kinase inhibitors for chemotherapy [259-264]. In a phase I clinical study of solid tumour cancers, canertinib in combination with docetaxel (which is effective against breast, NSCLC, advanced stomach, head and neck and metastatic prostate cancer) exhibited anti-tumour effects with complete response from patients with cervix uteri cancer and a partial response from NSCLC [263]. To evaluate the tumour response to treatments, the authors used the Response Evaluation Criteria for Solid Tumours [265] and were able to categorise the changes in tumour size to: complete response, partial response, stable disease, and progressive disease (where the appearance of new lesions is also considered) [263].

A phase 3 trial study in HER-2 overexpressed breast cancer patients [266] has shown that combining Herceptin, also known as trastuzumab, (an anti-HER-2 monoclonal anti-body), with standard chemotherapy was more effective than single agent treatments [266]. The antibody has previously been shown to exhibit synergistic effects with cisplatin and carboplatin [267, 268], docetaxel [269], doxorubicin, paclitaxel, methotrexate and cyclophosphamide [268-272] in *in vitro* and pre-clinical studies [273, 274].

In a pre-clinical study by Ciardiello et al., 2001, gefitinib (Iressa) an EGFR small molecule inhibitor enhanced the anti-tumour effects of taxanes and of platinum compounds [275]. Others have shown that a combination of BKM120 (a PI3K inhibitor) and Olaparib (a PARP inhibitor) may be effective in suppressing growth of ovarian cancer cells [240]. In view of the

effectiveness of such combinatorial approaches, this thesis will investigate the efficacy of combining two receptor tyrosine kinase inhibitors, canertinib and PHA665752 on growth, metabolism and adhesion of two advanced ovarian cancer cell lines in a novel 3 dimensional (3D) cell culture model to simulate the *in vivo* microenvironment.

1.7 Cell Signalling in Cancer Cells

Many cellular activities are regulated by an intricate system of interacting signal transduction pathways [276]. These include cell growth, proliferation, metastasis, and cell death. Signals from the extracellular environment are transmitted into the cells via transmembrane receptors or through the utilisation of membrane permeable ligands. Ligands can be growth factors, extracellular matrix components and/or cell adhesion molecules. The ligands bind to the extracellular domains of their specific receptors while other ligands bind to the cytosolic portion of the receptor by diffusing through the plasma membrane and binding to the receptors in the cytoplasm [277]. As a consequence, conformational changes of the receptors occur, resulting in dimerisation and subsequently autophosphorylation of the intracellular domains; e.g. the binding of c-MET receptor tyrosine kinase with hepatocyte growth factor (HGF) ligand [278]. Autophosphorylation in turn stimulates the transmission of specific signals that lead to the formation of different protein-complex molecules and their corresponding second small-molecule messengers [277]. The combination of the signalling cascades within the cells gives optimum cellular response that eventually cause cell growth, proliferation, and invasion [279]. Cell signalling in ovarian cancer envolves many types of receptors including nuclear hormone receptors, type 1 cytokine receptors as well as tyrosine kinase receptors. Nuclear receptors a intracellular proteins that are responsible for sensing of steroid and thyroid hormones and other molecues. They also participate in the regulation of the expression of specific genes. These receptors directly bind to DNA regulating the expression of adjacent genes. The focus of this thesis will be on tyrosine kinase receptors.

In cancer cells genes undergo mutations that may affect the signalling pathways either by upregulation or inhibition, for example amplification of EGFR and HER-2 receptor tyrosine kinases in ovarian cancer is thought to enhance tumour cell growth [280]. Amplification of the *HER-2* oncogene in breast and ovarian cancer have also been linked to tumour progression and constitutive activation of cell survival signal transduction pathways [281]. In follicular

lymphomas and chronic lymphocytic leukaemia, cancer cells may also evade programmed cell death by amplification of *BCL2* which in turn blocks apoptotic downstream signalling pathways [281].

1.7.1 Receptor Tyrosine Kinases

Receptor tyrosine kinases are a family of high affinity cell surface receptors for many cytokines, poly peptides, hormones and growth factors [218, 282]. Examples of receptor tyrosine kinases include epidermal growth factor receptors family (EGFR, HER-2, HER-3 and HER-4), mesenchymal epithelial transition factor (c-MET), insulin receptors (InR), and Platelet-derived growth factor receptors (PDGFR). They have been shown to not only be key regulators of normal cellular regulatory processes but also play a critical role in the development and progression of many types of cancers [283]. These receptors comprise three domains, an extracellular domain, a transmembrane domain and a cytoplasmic domain which represents the catalytic core and regulatory sequence [277, 284].

As detailed in section 1.9, once activated, either by binding to growth factors or by somatic mutation, receptor tyrosine kinases undergo hetero or homo dimerision and leading to conformational changes and auto phosphorylation and activating the cytoplasmic tyrosine kinase domain. Tyrosine phosphorylation is suggested to be a requirement for activation of these receptors to trigger cascades of intracellular signalling proteins that are recruited to promote cell growth, DNA replication and cell division [285]. Dysregulation of these receptors has been linked to the development of several types of malignancies. Therefore, these proteins have become a key target for cancer chemotherapy [218].

1.7.1.1 ErbB (Her-B) Family of Receptor Tyrosine Kinases

The ErbB family of receptor tyrosine kinases regulate cell growth, survival, proliferation, migration and differentiation. They are encoded by four genes: epidermal growth factor receptor (EGFR or ErbB1 (Her-1), human epidermal growth factor 2 (ERBB2 or HER-2/neu), ERBB3 (HER-3), and ERBB4 (HER-4). These transmembrane receptors have a molecular weight of between 170 and 185 kDa [286] and in the ovaries play a key role in the development of normal ovarian follicles and regulate the growth of the ovarian surface epithelium [287]. Aberrant catalytic activity that may occur through mutation or over

expression of these receptors can play a pivotal role in cancer progression [288]. This family of receptors have shown elevated expression in several types of cancer including NSCLC [289], breast [246, 248], prostate [290], head & neck [291], brain [292] and stomach cancer [293] as well as ovarian cancers [294].

EGFR was the first member of the tyrosine kinase receptor family to be identified [295]. It interacts either with another EGFR (homodimerisation) or with other members of the family HER-2, HER-3 and HER-4 (heterodimerisation) [286, 296]. Upon binding with its ligand, EGF, homo-dimerisation of EGFR (or hetero-dimerisation with other members of the family) occurs, and this promotes auto phosphorylation of the receptor and downstream signalling proteins [297]. ERBB2 has no known ligand and the activation is triggered via hetero dimerisation with other ERBB family members. However, the active kinase domain in ERBB3 facilitated the heterodimerisation with ERBB2 making it the preferred partner for ERBB2 sharing each other's active domains to activate transphosphorylation of the tyrosine kinases [298].

Amongst these signalling molecules are RAS/RAF/ Mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) which regulate cell adhesion, proliferation, differentiation, apoptosis, and metastasis [299] (**Figure 1.7**).

The Human Epidermal Growth Factor Receptor 2 (HER-2), also known as neu does not have a naturally binding ligand, but it may be constitutively activated or co-activated with EGFR to form a hetero- or a homodimer [84] and this partnering is suggested to inhibit EGFR degradation [300, 301]. This complex will auto-phosphorylate through its tyrosine kinase activity. It will then consequently activate the downstream signalling proteins [84, 302]. HER-2 receptors have been implicated as key drivers in cancer development and resistance to therapy in breast [303], colon [304], liver and ovarian cancer [305, 306] and their overexpression is a poor prognostic factor [86, 305]. Therefore, they have become a target for the development of anti-cancer treatments [307-309].

Abnormal expression of EGFR and HER-2 in cancer cells has been reported in 60% of ovarian cancers and in almost all histologic subtypes [310-312] and this has been found to be

correlated with a poor prognosis in several types of cancers [302, 305, 313, 314]. The mechanisms underlining the activation of EGFR include gene amplification, overexpression and deletion of the extracellular domain. The latter will induce constitutive activation of the receptor [315]. HER-2 mutations in such as increased copy number have been reported in many cancers including ovarian cancer are mostly expressed as gene amplification and protein overexpression [316, 317]. Since tumours tend to express high levels of EGFR, more EGF binds to the cancer cells promoting cell survival, proliferation [318, 319], and migration [320]. EGFR and HER-2 have therefore been suggested as potential targets for the treatment of ovarian cancers that express high levels of these receptors [309, 321].

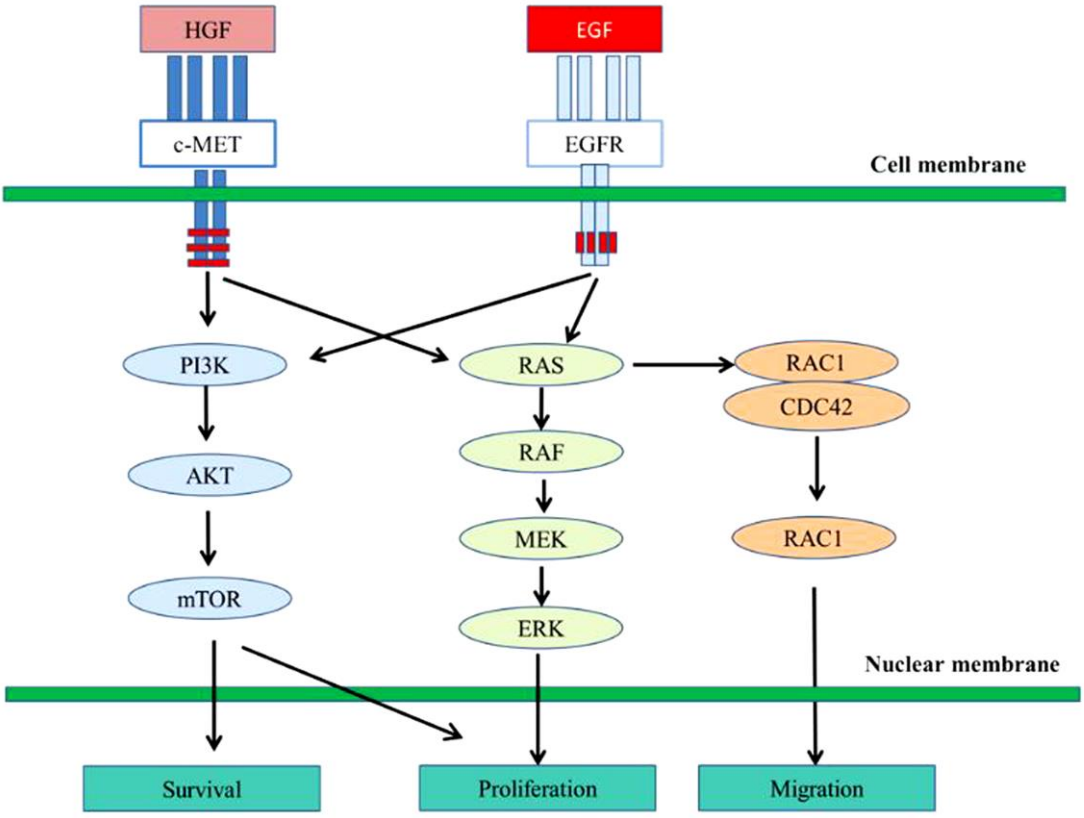


Figure 1.7 EGFR and c-MET signalling pathway. Dimerisation of EGFR and c-MET upon ligand binding leading to the activation of the relevant downstream intracellular proteins

involved in cell survival, proliferation and migration. Image adapted from Wang et al. with permission from the authors [322].

1.7.1.2 The c-MET receptor tyrosine kinase

The c-MET proto-oncogene is mostly found in epithelial cells [323-327], however it can be also be expressed in muscle cells, cytotrophoblasts, neurons, endothelial cells and macrophages [328-331]. The protein it encodes is an hepatocyte growth factor receptor that is associated with cancer progression and contains a tyrosine kinase domain that activates signalling pathways involved in cell proliferation, motility, adhesion, and invasion [332]. The c-MET signalling pathway plays a critical role in the growth, migration and spreading of ovarian cancer cells [323, 333-336]. The c-MET protein is comprised of two subunits; an α subunit of 145-kDa and a β subunit of 50-kDa (**Figure 1.8 a**) [337].

Following binding of HGF to its receptors, this binding initiates dimerisation of the receptors and autophosphorylation of tyrosine residues occur. First, the phosphorylation of residues Y1234 and Y1235 is then followed by residues Y1249 and Y1356 leading to the transmission of signals to downstream molecules. The overexpression of c-MET has also been correlated with poor prognosis in various cancers [338-343]. Reports suggest that high expression of c-MET is found in 11% -60% of epithelial ovarian cancers [333, 344] and some studies suggest that c-MET is overexpressed in the majority of ovarian cancers [288]. Treatment with c-MET inhibitors has been shown to significantly reduce tumor burden, ascites formation, protease activity and the number of peritoneal implants in an animal xenograft model [256, 345]. Despite this however, there are no reported notable changes of tumour size or angiogenesis. It has been suggested that c-MET overexpression is a prognostic factor in ovarian cancer and that targeting c-MET *in vivo* may inhibit peritoneal dissemination and invasion [334, 346].

The c-MET receptor can interact with EGFR/HER-2 suggesting that a mutual dependency may exist between these receptors [347, 348]. However, the exact mechanism by which potential co-activation may elicit the progression of ovarian cancer is still unclear. However, the fact that all three receptors can be overexpressed in ovarian cancer [29, 288, 349] suggests

a possible opportunity of a novel approach of treatment by combining two types of inhibitors. Targeting all three receptors may increase the longevity and potentially the survival rate of advanced ovarian cancer patients. These receptors normally have low expression in non-cancerous cells and so the inhibitors should have less of an impact on normal cells.

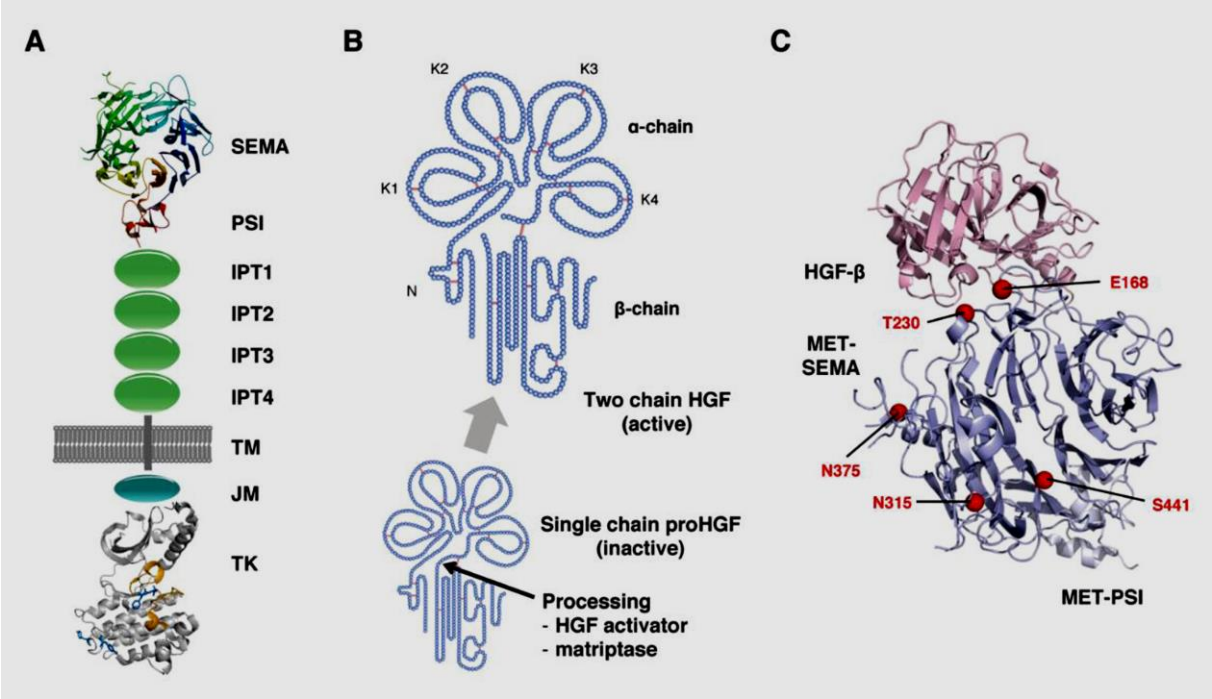


Figure 1.8. The structure of MET and its specific ligand HGF. a) The structure of MET, showing the phosphorylated tyrosine residues (Y1234, Y1235, Y1349 AND Y1356) in the tyrosine domain subsequently to HGF stimulation shown in blue. b) The structure of HGF before and after cleavage with matriptase, c) The crystal structure of the binding complex of HGF-MET. Red balls indicate the missense mutations found in cancer patients. Image taken with permission from the author Dr Donald Bottaro (NIH/NCI) [337].

1.7.2 Integrins

1.7.2.1 Integrins Structure and Binding Properties

Integrins are proteins that link the cytoskeleton with components of the ECM. They exist as heterodimers that are comprised of one α and one β subunit. They play an important role in numerous cellular processes including mediation of adhesion, migration, proliferation, differentiation and apoptosis. In addition to their adhesive capabilities, through conformational changes they are able to facilitate both inside-out and outside-in signalling across the plasma membrane. As detailed below they can play an important role in advanced stages of cancer.

In total there are 18 different integrin α subunits and 8 different β subunits that can dimerise non-covalently to form at least 24 distinct integrins which are capable of binding to different components of the ECM (as detailed in **Table 1.3**) [350, 351]. The α and β subunits each have a single transmembrane domain (TMD) comprised of at least 700 amino acid residues, a transmembrane helix, and a short cytoplasmic domain comprised of <50 residues (figure 1.8). The β4 integrin subunit differs in that it has a longer cytoplasmic domain of >1000 amino acid residues [352-354]. The cytoplasmic tale of integrin subunit β4 is believed to be the mediator of the association of the integrin with hemidesmosomes [355] in both in vitro cell culture and in vivo studies unlike β1 which is found located in the focal adhesion linked to actin filaments [356]. Integrins are able to undergo conformational changes and also to cluster with other integrins and receptors [357-359].

1.7.2.2 Integrin activation

Integrins can exist in bent, open extended and closed extended conformations (**Figure 1.8**). Previously, what was described as the bent conformation of integrins was thought to be the active form [360]. However, it was later shown that the bent structure is the inactive state and in order to be activated, the integrin needs to extend in shape and separate the "legs" in a process referred to as "switch blade" opening [361]. Takagi and colleagues demonstrated that the integrins in the inactive state mainly embrace the bent shape while activation of integrins with Mn^{2+} or cRGD will force the integrin to extend. They suggested that integrins are trapped in the bent shape by a disulphide bond which is broken upon activation to release the integrin [362]. Hynes and colleagues introduced the "angle-Poise" model suggesting that when inactive, the legs are bent close to the membrane while during activation the integrin will extend like an angle-poise lamp. This in turn will enhance the interaction with macromolecular ligands [363].

1.7.2.3 Integrins and cancer

It has been suggested that certain integrins may be highly expressed in cancer cells [364, 365] and this overexpression is associated with poor disease outcome and more aggressive metastasis [365-367]. Cell adhesion to the ECM is essential for cellular response to certain growth factors and vice versa these growth factors regulate the integrin-mediated cell adhesion and invasion [368]. Some studies have shown the synergistic effect and crosstalk at

the intracellular signalling pathway levels of the growth factor receptors and integrins [369, 370]. In particular this effect may involve interactions with receptor tyrosine kinases. Co-localisation studies suggest that integrins may associate with EGFR, HER-2 and c-MET [29, 371-374] and this may influence migration and metastasis through the mesothelial lining of the peritoneal cavity [370, 375, 376].

A pertinent example is the α6β4 integrin that is found in hemidesomosomes, cell-ECM attachment structures that link epithelial cells to the basal lamina [377]. The β4 subunit has a big cytoplasmic tail that is crucial for the assembly of hemidesmosomes [378]. Upon ligation, tyrosine phosphorylation of integrin α6β4 can fully activate the ERK and PI3K signalling molecules through the activation of integrin signalling pathway involving molecules such as Sch, Ras and Rho [379].

This subunit can be associated with EGFR/HER-2 receptor tyrosine kinases and the large cytoplasmic domain can become phosphorylated at several tyrosine residues upon activation of EGFR or binding to laminin 5 [380]. Thus a complex formed between EGFR and α6β4 which can cause the disassembly of hemidismosomes [173, 376, 380]. This disruption is thought to be a prerequisite for cell migration and invasion [376].

Ligand independent activation of the c-MET receptor tyrosine kinase has also been found highly dependent on integrins [381]. The crosstalk between c-MET and integrins is mainly regulated by many downstream signalling proteins including FAK [382].

Integrins commonly bind to components of the ECM at an RGD motif and as such studies on the inhibition of integrins have typically involved RGD containing peptides [383-385]. These have also been investigated with respect to cancer and a number of RGD-based (Arg-Gly-Asp) integrin inhibitors with low molecular weight have been have been approved for the treatment of several cancers [354]. Cilengitide, for example, is highly active against αvβ3 integrins with an IC_{50} = 0.6nM, which is in clinical trials for the treatment of several cancers [354]. However, these peptides show poor oral bioavailability and are usually quickly excreted.

Table 1.3. Integrin subunits and their suggested protein ligands. The table shows the 8 β and 18 α integrin subunits with different combinations of the two subunits and the different ECM protein ligands [377].

Integrin subunits		Ligands
β1	α1	Collagens, Laminins
	α2	Collagens, Laminins
	α3	Laminins, Fibronectins, Thrombospondin
	α4	Fibronectin, VCAM
	α5	Fibronectin
	α6	Laminins
	α7	Laminins
	α8	Fibronectin, Tenascin
	α9	Tenascin
	α10	Collagens
	α11	Collagens
	αv	Fibronectin, Vitronectin
β2	αL	ICAMs
	αM	Fibrinogen, ICAMs, iC3b
	αX	Fibrinogen, iC3b
	αD	VCAM, ICAMs
β3	αIib	Collagens, Fibronectin, Vitronectin, Fibrinogen, Thrombospondin
	αv	Fibronectin, Vitronectin, Fibrinogen, Thrombospondin
β4	α6	Laminins
β5	αv	Vitronectin
β6	αv	Fibronectin, Tenascin
β7	α4	Fibronectin, VCAM, MAdCAM
	αe	E-cadherin
β8	α6	Collagens, Laminins, Fibronectin

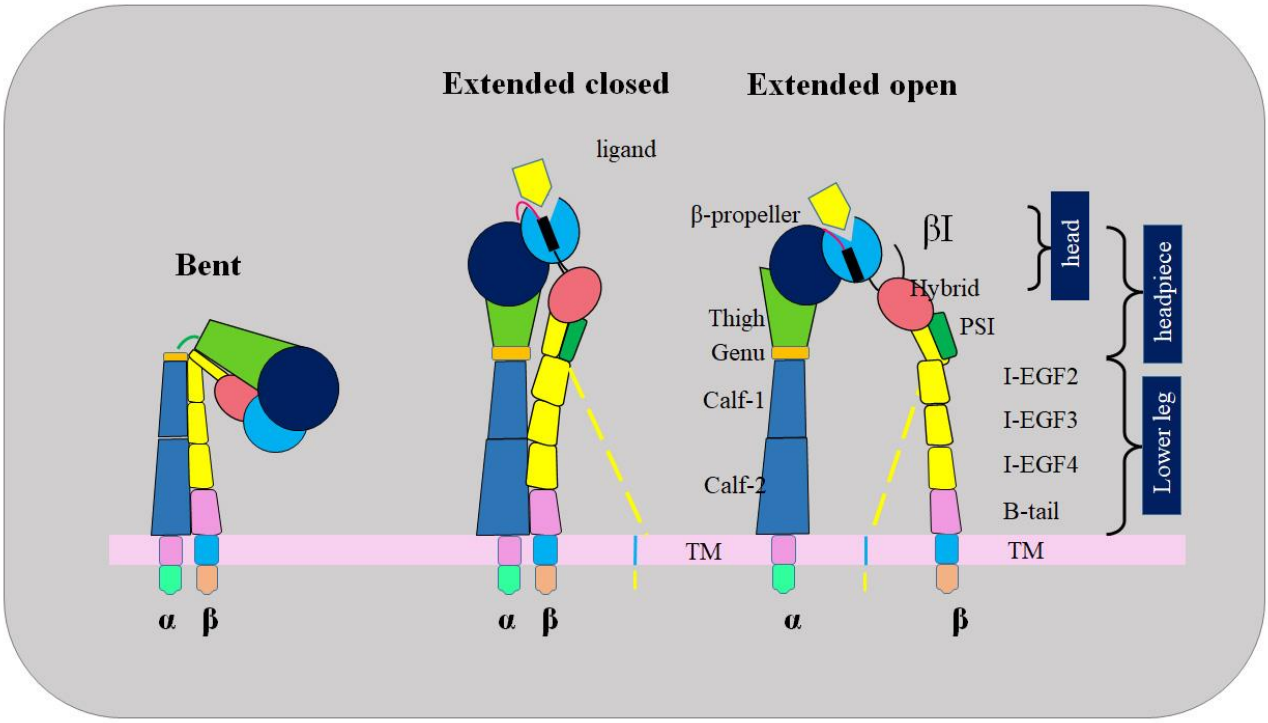


Figure 1.8. Integrin overview and complexes for ECM. The conformational changes of integrin. Dash lines show alternative conformations of the lower β leg, Calf1 and 2 are domains on the integrin subunit [386]. Image adapted and modified with permission from the correspondent author Dr. Donald P. Bottaro.

1.8 Growth Factors

Growth factors are polypeptides, which exert binding to their receptors and set off signal transduction cascades that regulate cell proliferation, motility and differentiation [387, 388]. If their receptors are present in abnormal levels, the growth factors can play a pivotal role in the establishment of METastatic microenvironment [389]. Examples of well-known growth factors are hepatocyte growth factor (HGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor (TGF)-beta [153, 390].

1.8.1 Epidermal Growth Factor (EGF)

Epidermal growth factor is a 6-kDa protein [391] of approximately 40 amino acid residues which contains 3 disulphide bonds [392, 393]. It is a potent mitogen that plays a crucial role in cell growth, differentiation, invasion and angiogenesis [394, 395]. EGF belongs to the same

family as transforming growth factor-α (TGF-α), heparin-binding EGF like growth factor (HB-EGF) and epidermal growth factor (EGF) [392]. These are derivatives of a glycoprotein precursor type-I that is composed of extracellular, transmembrane and cytoplasmic domains. To become biologically active these precursors undergo proteolytic changes [396].

EGF acts as a ligand for the ErbB family of receptor tyrosine kinases triggering autophosphorylation of several tyrosine residues, thus stimulating the phosphorylation of downstream signalling molecules [397]. The mechanism of EGF-EGFR binding is an asymmetric dimerisation [280, 398, 399]. The EGF-EGFR complex will bind to another receptor monomer (forming a homodimer or heterodimer) before binding to the second EGF. This in turn will cause conformational changes to the receptor prompting the kinase activity and autophosphorylation of the intracellular domains of the homodimers or heterodimers [397, 400]. EGF can bind to low and high affinity sites on cells with aberrant EGFR expressions [401]. High affinity binding is thought to be regulated by intracellular signalling proteins interacting with the intracellular tyrosine kinase domain of the receptor [402-404]. EGF has also been shown to stimulate the migration, invasion and metastasis of ovarian cancer cell lines through Matrigel [405].

1.8.2 Hepatocyte Growth Factor (HGF)

Also known as scatter factor, HGF is a potent mitogen derived from mesenchymal cells that is responsible for mediation of stromal-epithelial cell interactions and communication in several organs including the ovaries [406, 407]. It is thought to induce cancer cell invasion in the tumour microenvironment by working as a stromal cell-derived factor [408]. This polypeptide is originally secreted as single chain precursor which is then cleaved extracellularly by serine proteases into two subunits α and β (**Figure 1.8 b**) [409]. The α sub unit is a 69-kDa polypeptide and the β sub unit is a 34-kDa polypeptide linked with a disulphide bond [410-417]. It binds specifically with high affinity to the c-MET receptor through the N-terminal and first kringle domains (NK1) and low affinity through the β chain (**Figure 1.8 c**) [418]. Once bound to the receptor, HGF will activate a cascade of intracellular signalling pathways that regulates cell processes including cell growth, differentiation, migration, proliferation and survival [389]. In many types of tissues, including the ovary,

HGF is considered a mediator of mesenchymal-epithelial cell communications [406, 407, 419].

The ovaries regulate the expression of HGF through estradiol and Human Chorionic Gonadotropin (HCG) [406]. It is also pivotal for organogenesis in the early developing embryonic stage as well as tissue repair from organ diseases later in life [417, 420]. Ovarian cancer cells secrete HGF which can stimulate peritoneal implantation by inducing mesothelial-mesenchymal transition of the peritoneum [389, 418]. Expression of HGF was reported in normal ovarian cells of epithelial origin and benign ovarian tumour cells [421], and higher expression was found in ovarian carcinoma cells [422]. In an immunocytochemistry analysis of tissue from ovarian cancer cases, researchers found high expressions of HGF [175]. This high expression suggests an important role in the onset of ovarian cancer and tumour progression by stimulating the abnormal cell proliferation and metastasis [389]. Other cancer cells including breast [423] and lung [424] cancers are also known to secrete high levels of HGF.

1.8.3 Vascular Endothelial growth factor (VEGF)

Previously, VEGF was recognised as an endothelial cell-specific mitogen that played a crucial role in physiological and pathological angiogenesis and vascular permeability [425, 426]. However, it was later discovered that VEGF can be a key driver in cancer cell migration, invasion and survival of tumour cells and is associated with poor disease outcome [427]. The potent pro-oncogenic cytokine stimulates vascularisation and angiogenic processes [428, 429]. VEGF was shown to target not only endothelial cells, but rather other cells in the tumour microenvironment including immune cells [430].

The ovaries, being distinct from other endocrine organs, require angiogenesis within their various compartments [431]. This means that the production of VEGF is crucial for normal reproductive function [432]. Also associated with ovarian pathogenesis, VEGF levels in ovarian cancer-induced malignant ascites are significantly higher compared to those in non-malignant ascites [433]. Ovarian tumours are known to be highly vascularised neoplasms predominantly reliant on VEGF-mediated angiogenesis [434]. Hence VEGF has been shown

to play a critical role in the peritoneal dissemination of ovarian cancer and the development of malignant ascites [153].

VEGF binds to vascular endothelial growth factor receptors (VEGFR), which are receptor tyrosine kinases found overexpressed in most solid malignant tumours [435]. VEGF is a critical molecule for angiogenesis and its specific receptor, VEGFR2 is a key regulator of the proliferation, differentiation and invasion of endothelial cells and most importantly microvascular hyper permeability, the first step towards angiogenesis [436]. In addition to inducing vasculature, VEGF produced by tumour cells works in an autocrine loop by binding to the highly expressed VEGF receptors on tumour cells [430]. These receptors are present at low levels in normal cells while in cancer cells they have high expression [437]. This means that more VEGF will bind to its receptors on cancer cells therefore promoting cell survival [438], proliferation [318, 319], and migration [439]. Consequently, treatment schemes that involve inhibition of VEGF may be beneficial for ovarian cancer patients.

1.9 Ovarian cancer cell lines and molecular subtypes

The development of *in vitro* tumour cell cultures has greatly enhanced research into cancer. Tumour cell lines have been developed and used as models to increase our understanding of the various aspects of disease progression. However, the drawback of using cell lines is that they may not accurately represent the *in vivo* situation. Nevertheless, the *in vitro* models have been the first line of tools to investigate the response of tumour cells to anti-cancer drugs.

In the process of cell line selection for preclinical settings that model newly-discovered molecular sub-groups, the integration of gene expression data sets is of most importance.[440]. This is of specific significance in any ovarian cancer in vitro research given the heterogenous nature of the disease.

One of the important characteristics of ovarian cancer cells is the diversity of mutations that can accumulate leading to their development. These define the molecular subtypes and the behaviour of these cells . Low grade ovarian serous carcinomas, for example, are categorised as type I ovarian tumours which have mutations in *PIK3CA*, *PTEN*, *KRAS*, *BRAF* and *CTNNB1*. Cell lines that express these mutations are known as type I tumour cells. High grade

serous ovarian carcinoma cells have *TP53* and *BRCA1/2* mutations and fall under the type II molecular subtype.

However, unrelated model systems do offer an incentive for investigation of biochemical processes but particular cellular context needs to be applied to assess relevant therapeutic strategies. The HGSC cell lines may not have the mutations in RTK [441].

Recently, Ince and colleagues [442] characterised 25 ovarian cancer cell lines that phenocopy primary tumours and highlighted the importance of culture medium in the use of cell lines. In their study they collected 90 tumour samples from 67 patients with 44 serous, 2 clear cell, 2 endometrioid and 3 mixed mullerian cancers, in addition to 16 specified adenocarcinomas. They proposed that the cell culture medium, being a rich environment with artificially added ingredients such as tissue extracts, antibiotics, serum, and feeder layers may induce biological variability and confound cytostatic assays. In light of this they developed a highly efficient cell culture system that was thought to preserve the original tumour phenotype with over 80 ingredients for providing essential nutrients for maintaining basic cellular metabolism [443]. They concluded that given appropriate condition it was possible to establish a comprehensive ovarian carcinoma cell panel from the tumours of advanced ovarian cancer patients, and established a culture medium that highly maintains the phenotype of the original tissue.

The cancer genome atlas (TCGA) published a molecular description of over 500 tumour tissue types and the broad-Novartis Cancer Cell Line Encyclopaedia (CCLE), introduced a systemic evaluation of the tumour types and cell lines' diverse molecular heterogeneity regarding the level of DNA copy number and the intensity of mutations [80, 444]. Based on these two reports, Domcke and colleagues have evaluated selected ovarian cancer cell lines and their mimicking of tumour models [445]. Focusing on high grade serous ovarian carcinoma cell lines, they showed that there were several cell lines, which have been extensively used in ovarian cancer research as models of HGSOC but that these they may have actually originated from a non-ovarian origin and thus may be a poor representative of HGSOC.[445].

Among the cell lines indentified by Domcke et al. were SKOV-3 cells which have been used in this thesis. SKOV-3 cells were shown to have a flat copy-number profile, null *P53*, and other mutations such as *ARIDA, BRAF, PIK3CA and PTEN*. While it can be argued that these molecular profiles do not match the HGSOC profile [441, 445, 446], SKOV-3 cells are a close representative of the ovarian clear cell carcinoma subtype, which shows an aggressive phenotype and for which currently there is no effective chemotherapy that can improve the survival of patients with ovarian clear cell cancer.

The basis for using SKOV-3 in this thesis was the aberrant expression of the receptor tyrosine kinases, EGFR, HER-2 and c-MET which makes this cell line a good candidate *in vitro* model to study these receptors in terms of inhibition with TKIs, as well as their association with integrins and roles in cell adhesion. Tumour suppressor gene (*TP53*) is often deregulated in the tumorigenesis of many tumours including ovarian tumours [447] and is proposed to play a crucial role in the development of ovarian cancer [447].

In contrast, OVCAR-5 cell lines show *P53* mutations with low expression of HER-2. Moreover, both cell lines are able to form 3 D cell clusters and spheroids that mimic the floating clusters and aggregates that are seen in malignant ascites from ovarian cancer patients, and thus these two cell lines were chosen as the molecular subtypes for this study.

1.10 3-Dimensional microenvironment

In many studies 2-dimensional cell culture models have been used for *in vitro* cell culture research and these have played a critical role in the development of anti-cancer drugs [448]. However, the 2 D monolayers may not faithfully replicate the properties and behaviour of cancer cells in the body with the result that the cellular responses to treatments *in vitro* were not replicated in the clinical setting [449]. There is evidence to suggest that 3-dimensional cell culture models may more accurately mimic the biological and physical properties of the cells in the *in vivo* microenvironment [450, 451]. Gene regulation and protein expression in cells that are part of a 3 dimensional cell suspension have been shown to be distinct from those in monolayer cell cultures [452, 453]. Additionally there are many other features that impact on cell behaviour such as mechanical forces and cell-cell and cell-ECM interactions which cannot be replicated in a monolayer [450, 454]. Recently, Lee and colleagues [450] described

several differences in cell morphology, adhesion, proliferation, division, heterogeneity and cell culture environment between free floating aggregates and monolayer cells [450]. These were:

1) The 2D cells were flat and stretched while the 3D cultured cells maintained normal cell shape.

2) Cellular adhesion occurred rapidly in 2D flat cells while the same process was much slower in the cell suspension.

3) 2D cells showed a uniform proliferation rate that was faster than that of *in vivo*, however, 3D aggregates showed a closer approximation to the *in vivo* rate

4) 2D cells exhibited partial apical-basal polarisation whereas 3D aggregates displayed a more precise cell polarization.

5) 2D cells were generally in the same state while cells in 3D suspensions were in distinct states such as quiescent, proliferating, apoptotic, necrotic and hypoxic cells and finally,

6) Flat cells were exposed in equal measure to the growth media/treatments whereas this was not the case in 3D cultures where each cell essentially had their own microenvironment which mimics that of a tumour. [450].

Furthermore, with respect to the current thesis ovarian cancer cells often form 3 dimensional cellular aggregates floating in the ascitic fluid and these are the major source of implantation and secondary tumour growth [455]. Thus it seems likely that studies of the effect of drugs on ovarian cancer cell lines should incorporate the use of 3D cell culturing techniques.

1.11 The proposed research

An anticancer drug, which inhibits the activities of cancer cell clusters/compact aggregates so preventing growth and adhesion onto peritoneal surface of internal organs, may have a significant clinical impact on the survival rate of women with ovarian cancer. EGFR inhibitors including gefitinib, erlotinib, and lapatinib have been used to fight several types of cancers and have shown that the use of a single agent inhibitor has a notable reduction of tumour progression and can increase survival rate of selected patients with minimal side

effects [456]. However, EGFR inhibitors have shown only modest clinical benefit in ovarian cancers [457]. Moreover, a combination of EGFR inhibitors with other cytotoxic agents has been found to be ineffective against ovarian cancer [458]. This lack of effect may reflect the possibility that the cancer cells may switch to alternative pathways, possibly by activating the c-MET receptors in order to overcome the inhibition of EGFR inhibitors.

Given this, the effect of inhibition of c-MET will be investigated alongside EGFR inhibition in this project. The concept is that one-drug for one-target is not likely to be effective in ovarian cancer. The idea of using combined two or more targeted drugs, which exhibit inhibition of a broad range of receptors would be a more effective treatment. Canertinib is an irreversible pan-EGFR inhibitor. It has been shown to exert inhibitory effects on cancer cells which have high levels of EGFR and HER-2 proteins [247]. The rationale for using canertinib is that the drug can bind to both EGFR and HER-2 receptors permanently. Therefore, cellular activities of cell clusters/compact aggregates via EGFR/HER-2 receptors can be significantly reduced. Additionally the interaction of EGFR/HER-2 with the tyrosine kinase c-MET receptor needs to be explored and may introduce new aspects for treatments in advanced ovarian cancer.

There are two important aspects of ovarian cancer that are of particular interest for this study. Firstly, the association between EGFR, HER-2 and c-MET and the survival process of the ovarian cancer cell lines in routine cell culture media supplemented with specific growth factors and in the ascitic fluid supplemented media will be investigated. Two advanced ovarian cancer cell lines will be cultured in non-adherent conditions to generate cell clusters/compact aggregates that mimic ascitic cell clusters. Secondly, the adhesion step which represents the early events of cancer cell attachment to the surface of the peritoneal membrane. The inhibitors of interest in this research are Canertinib (EGFR/ HER-2 inhibitor), and PHA665752 (c-MET inhibitor).

1.12 Hypotheses

- The combination of two receptor tyrosine kinase inhibitors (canertinib and PHA665752) will compromise cell growth, cellular metabolism, adhesion via mediating the activation of and EGFR, HER-2 and c-MET.

- Ascitic fluid will affect growth activity via EGFR, HER-2 and c-MET mediated activation and inhibitors will affect the activity.

- EGFR, HER-2 and c-MET will play a role in cell adhesion process, which can be inhibited by blocking the receptors.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell lines, media and chemicals

Two human ovarian adenocarcinoma cell lines were used in this study, OVCAR-5 and SKOV-3, which were kindly provided by Dr. Kenny Chitcholtan, (Department of Obstetrics and Gynaecology, University of Otago, Christchurch). Cell lines were subject to STR and mycoplasma testing. Cell lines were as reported and there was no mycoplasma contamination. These cells were maintained in Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEMF-12) (GIBCO®, Thermo Fisher, Auckland, New Zealand) and Minimum Essential Media (MEM) (GIBCO®, Thermo Fisher, Auckland, New Zealand), working media, respectively. For the base media, MilliQ water was autoclaved prior to the media preparation process; one sachet of medium powder (DMEMF-12 or MEM) was dissolved in the appropriate volume of the autoclaved MilliQ water, then 0.37% w/v and 0.22% w/v of Sodium bicarbonate ($NaHCO_3$) was added to DMEMF-12 and MEM, respectively and the pH for the solution was adjusted to 7.2 using 0.1 N NaOH. Media were filter sterilised in a laminar flow cell culture hood into 1 L sterile bottles and kept at 4°C prior to use. The final pH of media was 7.4 after filtering.

Fresh working media were prepared by supplementing the base media solution with 5% (v/v) Foetal Bovine Serum (FBS) (GIBCO®, Thermo Fisher, Auckland, New Zealand,), 2 mM Glutamax (GIBCO®, Thermo Fisher, Auckland, New Zealand, New Zealand), PenStrep antibiotics comprising 100 units/mL penicillin, 100 µg/mL streptomycin (GIBCO®, Thermo Fisher, Auckland, New Zealand) and 2 µg/mL Fungizone (Sigma). The working media were stored at 4°C prior to use.

Both cell lines were cultured in 50 or 250 mL cell culture flasks containing the working media and incubated at 37°C in a 5% CO_2 humidified incubator. Cells were allowed to grow until near full confluence by monitoring them with a light microscope with the media changed every second day. Once cells were confluent, cells were sub-cultured by discarding the media and washing the cells with 1x sterile Phosphate Buffer Solution (PBS) previously prepared from a stock of 10x PBS . One litre of 10x PBS was prepared with 1.4% Na_2HPO_4, 9% NaCl, 0.2% KCl, the pH was adjusted to 7.4 with NaH_2PO_4 and topped up to 1000 mL with MilliQ water. 1x PBS was prepared using 100 mL of 10x PBS, diluted to 1000 mL with MilliQ water

and autoclaved for cell culturing purposes. Cells were then centrifuged to discard PBS solution and reconstituted in working medium then counted and sub-cultured in a new flask.

2.2 Generating 3D Cell Cultures

In order to set up 3D cell cultures, confluent cells were incubated for 20-30 minutes with 1x trypsin-ethylene diamine tetra acetic acid (trypsin-EDTA) (Thermo Fisher, Auckland New Zealand) to detach them from the culture flask. Once detached from the flask, cells were centrifuged at 400 g for 5 minutes, and the supernatant was discarded and cell pellets were re-suspended in the appropriate working media. A cell count was performed using a haemocytometer and an Olympus CK40 light microscope in order to calculate the volume of cell suspension needed to obtain $1x10^5$ cells/mL in each well of a 24 well plate.

To generate the 3D cellular aggregates, each plate was pre-coated with 300 µl of 24 mg/mL of poly-hydroxyethylmethacrylate (poly-HEMA) (Sigma, Auckland New Zealand). This was warmed prior to application. The coated plates were allowed to dry overnight on an orbital shaker at 37°C, and then were then left at room temperature to dry over night before washing them with 1x PBS prior to usage. The poly-HEMA prevents the cells from adhering to the bottom of the wells; hence cells tend to cluster forming 3D clusters and compact aggregates. A concentration of 24 mg/mL of poly- HEMA solution was prepared by mixing 2.4 g of poly-HEMA with 100 mL of 95% ethanol. The solution was heated to 72°C with continued stirring until fully dissolved.

The volume of cell suspension was determined in order to obtain $1x10^5$ cells/mL in each well. The final volume in each well was 1 ml. Approximately 500 µL of the remaining cell suspension was added into a new culture flask with fresh working medium for cell culture maintenance. Cell culture plates containing cancer cells were incubated for 6 days to generate cell clusters and aggregates. Cell media were refreshed with fresh media every second day. Prior to experimental stimulations, cells were maintained in a serum free medium (SFM) for an additional 24 hours (prepared as working medium minus the FBS). When changing medium, plates were allowed to rest at an angle for approximately five minutes to allow the floating cell clusters/compact aggregates to settle down before aspirating the medium in order to help to prevent any loss of cells.

2.3 Making 2D Cell culture (cell monolayer)

Some experiments with 2D cell cultures were performed for comparative purposes. For these experiments, cells were cultured in 24 well plates without the poly-HEMA, so that the cells would adhere to the bottom of the wells. Apart from that, culturing procedures were similar to those of 3D cell cultures.

2.4 Treatments with growth factors and tyrosine kinase inhibitors (TKIs)

2.4.1 Growth factor stimulation

In order to investigate the role of growth factors in the activation of 3D cellular clusters/ compact aggregates, cells were exposed to epidermal growth factor (EGF), (Thermo Fisher, Auckland, New Zealand), hepatocyte growth factor (HGF), (Thermo Fisher, Auckland, New Zealand), or a combination of both growth factors (GF). Two concentrations of growth factors were used 20 ng/mL (a concentration used in most of the published literature) and 0.2 ng/mL (a physiologically relevant concentration, found in ascitic fluids of patients with advanced ovarian cancers). OVCAR-5 and SKOV-3 ovarian cancer cell lines were cultured in 3D clusters and aggregates for 6 days in working media, and then 24 hours in SFM before the addition of SFM supplemented with growth factors. Cells were maintained in growth factor stimulating conditions for 48 hours. Clusters/ compact aggregates were collected and assessed for cellular metabolism, using Alamar blue staining and a cell count was performed.

2.4.2 Treatment with tyrosine kinase inhibitors (TKIs)

Following the generation of 3D cellular clusters/ compact aggregates, as described above (see section 2.1.1.), cells were treated with two TKIs, canertinib, also known as CI-1033 (LC laboratories Massachusetts, USA) and PHA665752 (Sigma, Auckland, New Zealand), either singly or in combination for 48 hours before analysing for cellular metabolism and cell number.

In order to determine the lowest effective concentration of each inhibitor, concentration dependent experiments were initially performed with each inhibitor before conducting the combination treatments.

All samples were normalised against the vehicle (which in this case is DMSO, the solvent used to dissolve the drugs), by calculating the amount of DMSO in each sample according to the volume of the treatment added and normalising the other samples by adding the difference in volumes. For the initial concentration dependent experiments, the concentrations of canertinib were 0.5, 1.0, 2.0, 3.0 and 4.0 µM, and the concentration of PHA665752 were 0.1, 0.25, 0.5, 1.0 and 2.0 µM. Three replicates of each treatment within an individual experiment were carried out and the experiments were repeated at least 3 times for each cell line. Cellular clusters/ compact aggregates were analysed for cell morphology, cellular metabolism and cell number.

2.5 Analysis of cluster and aggregate morphology

For analysis of the morphology of cellular clusters and aggregates, the size of the clusters, their appearance and any changes induced by the treatments were monitored. After culturing for 6 days, cellular clusters of OVCAR-5 and compact aggregates of SKOV-3 were imaged using an inverted light microscope equipped with a digital camera, using a 10x/0.25 N.A. objective lens (ACCU-SCOPE" 3032). After completion of inhibitor exposure, cells in clusters and aggregates were harvested and digested with 1x trypsin-EDTA to obtain single cell suspensions, and cells were counted using a heamocytometer to assess cell number.

2.6 Assay of Cellular metabolism using Alamar blue dye

To assess cellular metabolic activity following the treatments, 100 µl of 400 µM Alamar blue dye (Resazurin, Thermo Fisher, Auckland, New Zealand), was added to each well containing cells and 1 ml media, and these were allowed to incubate in a CO_2 atmosphere at 37°C for 4 hours. Alamar blue is a resazurin blue dye that is metabolised by actively respiring cells and the blue dye is reduced to resorufin (a pink compound), which is then secreted into cell culture media. After 4 hours incubation, a 200 µL of cell suspension medium was transferred into a 96 well plate and the difference of absorbance of Alamar blue dye at 570 and 600 nm was measured using a microplate reader (Spectra Max® M5, Molecular Devices).

The difference in absorbance between 570 and 600 nm was used as an indicator of cellular metabolism.

2.7 Measurement of cell number

After the cellular metabolism assay was assessed, cells were collected to determine cell numbers. Cells were washed from the Alamar blue dye with 1x PBS at least two times before trypsinisation with 2x trypsin-Ethylene diamine tetra acetic acid (EDTA) and incubation at 37°C for approximately 20 minutes before the number of cells was counted using a heamocytometer.

2.8 Vascular Endothelial Growth Factor (VEGF) detection via Enzyme-Linked Immunosorbent Assay (ELISA)

Following the growth experiments, the clusters/ compact aggregates were centrifuged and the supernatant (media) was collected for VEGF secretion analysis. The protocol from the manufacturer was applied as follows: 96 well ELISA plates (Nunc, Thermo scientific, Illinois, USA) were pre-coated with mouse anti-human VEGF capture antibody (diluted in 1x PBS) and incubated overnight at room temperature. Next day the wells were washed using washing buffer (0.05% Tween-20 in 1x PBS) for four times and dried each time by tapping the plate on drying tissue before incubation with VEGF reagent diluent (1% BSA in 1x PBS) for 60 minutes at room temperature. The wells were then washed x4 before loading the samples and standards (serial dilution of standards was with 1x PBS) and incubating at room temperature for two hours. The plates were then washed four times before adding biotinylated goat anti-human VEGF detection antibody (this was diluted using reagent diluent) and incubated for further two hours a room temperature. Next step was washing x4 before incubating in the dark for 20 minutes with streptavidin-HRP (diluted with reagent diluent). Final washes x4 were executed before adding ultra TMB-ELISA and incubating for 7 minutes. Stop solution was added to stop the reaction (2 N NH_2SO_4) and the absorbance was measured using the microplate reader at 450 nm.

2.9 Assay of total Protein concentration produced by cancer cells

To investigate the effects of the treatments on cell growth at cellular levels, protein concentrations of the treated samples were also assessed and compared to the untreated controls. Cells were cultured for 6 days in working medium, then 24 hours in SFM before incubating with the inhibitors for 48 hours. Cells were then washed with ice cold 1x PBS, and lysed using either radio-immuno precipitation assay lysis buffer, RIPA (made up with 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.1% (w/v) SDS, 10% (v/v) glycerol, 1 tablet protease inhibitor), or 0.1% SDS (0.1% SDS dissolved in 1x PBS), or modified 0.1% SDS buffer (0.1% SDS was dissolved in 10 mL of 10x PBS, 10 mL Glycerol, 0.183 g EDTA, 2 tablets of protease and topped up to 100 mL of MilliQ water). Cells were mixed and left on ice for 30 minutes. Cellular components were fractionised by centrifugation at 6700 g for 10 minutes and the supernatant was transferred into new Eppendorf tubes before determining the protein concentration of each sample using a Micro-BCA^{TM} Protein Assay Kit (Pierce Thermo Fisher Scientific, Rockford, IL, USA). A stock solution of 2 mg/mL bovine serum albumin (BSA) was prepared and aliquoted in small Eppendorf tubes and stored in -20˚C until use. A serial dilution of standard protein BSA was subsequently prepared according the manual provided by the BSA assay kit. Standards were run with all experiments individually and the optical density was measured and plotted against the protein concentration. A linear trend line with the equation was applied and protein concentrations were calculated accordingly, as described by the manual.

2.10 Immunoblotting Analysis for protein expression

To determine the expression of EGFR, HER-2 and c-MET, their phosphorylated forms, p-EGFR, p-HER-2, p-MET and their downstream signalling molecules, PCNA, Akt, p-Akt, ERK, and p-ERK Western blotting was performed on the protein in cellular lysates. Following determination of the protein concentrations, the volumes of cell lysates to be loaded on the gels were calculated in order to obtain a total of 10 µg of protein. Protein lysates were denatured by boiling with 0.2% v/v of Laemmli 5x sample buffer (0.2% (v/v) bromophenol blue, 25% (v/v) glycerol, and 14.4 mM 2-mercaptoethanol in Tris-HCl pH 6.8), for 10 minutes. All samples were left on ice for 30 minutes before loading into freshly made

sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) gels that were 1 mm thick.

For each individual gel preparation a 10% SDS-separating gel was made using 2.8 mL MilliQ water, and 1.66 mL Solution A (30% (v/v) acrylamide, 25 mL of 10% $(NH_4)_4S_2O_8$), 1.25 mL solution B (75 mL of 2 M Tris-HCl pH 8.8 (24.2% Tris-base in MilliQ water, pH adjusted to 8.8) for final concentration of 1.5 M) and 2.5 µl TEMED. The mixture was loaded into the gel cassettes, topped up with MilliQ water to keep it hydrated, and incubated for 60 minutes at 37°C. Then a 7% SDS-PAGE stacking gel was made using 1 mL MilliQ water, 0.5 mL solution C (50 mL of 1 M Tris-HCl, pH 6.8 (12.1% of Tris-base in MilliQ water, pH adjusted 6.8) for a final concentration of 0.5 M.), 0.5 mL Solution A, 20 µL of 10% Ammonium per sulphate $(NH_4)SO_4$, and 2.5 µl TEMED. The mixture was added immediately to the separating gel and a 10 well comb was inserted before incubation for another 30 minutes at room temperature. The SDS-PAGE was run for 3-4 hours at 120 Volts using freshly made up running buffer (0.3% Tris, 1.4% glycine, 0.1% Sodium dodecyl sulphate (SDS), all dissolved in 1.0 L of MilliQ water), to separate the proteins. Precision Plus Protein™ standard (BioRad, Hercules, USA) and MagicMark (MagicMark™ Western Protein Standard Life Technologies, New Zealand) were used as ladders. Once bands were separated, proteins were electro-blotted onto Poly Vinyl PVDF membrane (Immun-Blot®PVDF Bio-Rad USA) for 15-30 minutes using a BIO-RAD Trans-Blot® Turbo™ Transfer system. The PVDF membranes were incubated with methanol for 5 minutes before washing with MilliQ water and soaking with the transfer sponges in freshly made transfer buffer (0.19% Tris, 0.9% glycine dissolved in MilliQ water) at least 15 minutes prior the transfer.

Membranes were then blocked with the specified blocking solutions, 5% skim milk or 1-4% BSA, dissolved in Tris-buffered Tween saline TBS-T (10% of 2 M Tris-HCl, pH 7.5 (24.3% Tris-base in MilliQ water adjusted to pH 7.5), 0.82% Sodium chloride (NaCl) in MilliQ water and autoclaved before adding 0.1% (v/v) of Tween-20). Blocking was carried out for 1 hour before incubation with the primary antibody solutions overnight at 4°C with gentle shaking. Primary antibodies were diluted at a range of 1:500, 1:1000 or 1:2000 with 50% (v/v) of TBS-T and the appropriate blocking buffer (all primary and secondary antibodies are described in **Table.2.1.**). The next day, the membranes were washed with TBS-T 4 times for 10 minutes

before adding the secondary antibody and incubating at room temperature for 90 minutes on an orbital shaker followed by washing with TBS-T 4 times for 10 minutes.

Secondary antibodies were diluted 1:5000 or 1:10000 with 50% (v/v) TBS-T and the specified blocker. Membranes were developed using Clarity™ Western ECL Blotting Substrates detection kit (Bio Rad, USA). Protein bands were visualised and densitometry analysis was performed using Alliance 4.7, Unitec software (Cambridge, UK). Densitometry readings were based on median bands from raw data images and normalised against the band intensity of GAPDH as a reference protein.

2.11 Detection of cellular proteins using Immunofluorescence

OVCAR-5 and SKOV-3 cells were cultured to generate cell clusters and aggregates for 6 days. The media were refreshed every second day. Cell clusters and aggregates were collected and fixed with ice cold 50% (v/v) acetone/methanol solution. OVCAR-5 cell clusters were washed twice with ice cold PBS, pH 7.4 and re-suspended in 200 µl cold PBS. Clusters were then mounted on poly-lysine coated microscope slides left to dry at 37°C for 20 minutes before proceeding with the main procedure. Prior to frozen sectioning, SKOV-3 aggregates were washed with PBS pH 7.4 and stained with 1% aniline blue dye solution (Sigma-Aldrich LTD, New Zealand) for 15 minutes. The aniline blue dye binds to proteins on cell aggregates and makes aggregates more visible when a frozen section was cut. Cells were washed 2 times for 10 minutes each time with ice cold 1x PBS before they were embedded in a liquid CryO-Z-T solution, OCT (Ted Pella Inc., USA), an embedding medium used to aid sectioning of frozen tissue samples in a cryostat. The liquid OCT blocks were frozen at -80°C for at least 24 hours. Seven µm thick slices were sectioned from the block of frozen OCT using a CM186UV Cryostat, (Lieca BIOSYSTEM, Deutschland) and were placed on an appropriately labelled Superfrost plus slides (Menzel-glaser, Germany). Six slices per sample were collected, and stored at -20°C until analysis. Both cell lines were blocked with 4% BSA in 1x PBS for 1 hour at room temperature before incubation overnight with a 1:200 dilution of primary antibody at 4°C. Next day, cells were washed twice with ice cold PBS (pH 7.4) for 10 minutes each time and further incubated with a 1:500 dilution of secondary antibody goat anti-rabbit IgG conjugated with FITC for 60 minutes in a 37°C incubator.

The secondary antibody solution was removed and 500 µL of 10 µg/mL of (4`, 6-diamido-2-phenylindole (DAPI, Thermo fisher NZ), a fluorescent dye that binds to DNA, was added to the cells and left for 20 minutes in the dark. Cells were then washed twice with ice cold 0.1% Tween-20 in PBS pH 7.4 for 10 minutes each wash then mounted with ice cold anti-fading solution (2 mg/mL p-phenylenediamine in 80% glycerol, pH 7.8). Immuno fluorescent staining was carried out on both cell lines with anti-EGFR (SC120), anti-HER-2 (SC-7301), and anti-c-MET (SC10) primary antibodies (Santa Cruz). Fluorescent images were captured using an epifluorescence microscope with a 40x/1.3 N.A. oil/DIC objective lens (AxioVision 4.5. Apotome software, Carl Zeiss, Oberkochen, Germany).

2.12 Ascitic fluids from ovarian cancer patients

Ascitic fluids from three advanced ovarian cancer patients were collected during debulking surgery, and they were referred as P1, P2, and P3 (kindly donated by patients from Gynaecology Department at Christchurch Women's Hospital). Patient's consents were obtained by Dr Kenny Chitcholtan prior to collection of ascitic fluids. The study protocol to obtain ascitic fluid from ovarian cancer patients was approved by Health and Disability Ethics Committees (Wellington, New Zealand). The Ethics reference number is URA/12/06/019/AM01

Cellular components in ascitic fluids were removed by centrifugation and cell free ascitic fluids were kept at -80°C for further studies. Ascitic fluids were aliquoted into 50 ml tubes and kept in -20°C freezer until use. The history of patient information was included in the Appendix.

2.13 Effect of ascitic fluids on cell growth and cellular uptake of PHA665752

In order to investigate the effect of ascitic fluids on cell growth and the cellular uptake of PHA665752, cells were exposed to a variety of conditions as summarised in **Figure 2.1**.

The c-MET inhibitor, PHA665752 has a light green colour, which has fluorescent characteristic during FACS analysis (Chitcholtan, personal communication). This property made it possible to detect the cellular uptake of PHA665752 and determine whether the

inhibitor is entering the cells in the presence of ascitic fluids or in bovine serum albumin (BSA) which was used as a representative of human serum albumin. This would in turn give an insight of the effectiveness of the inhibitors on ovarian cancer cell clusters and compact aggregates in the presence of ascitic fluids or BSA. OVCAR-5 clusters and SKOV-3 compact aggregates were treated with PHA665752 in the presence of 50% v/v of patient's ascites and incubated for 48 hours (ascites used in literature is approximately 5-10% but a more concentrated ascites could closer mimic the *in vivo* microenvironment so 50% was chosen). Cells were then washed with 1x cold PBS, and trypsinised, before measuring fluorescent cells, as an indicator for cellular uptake using flow cytometry (Beckman & Coulter, USA).

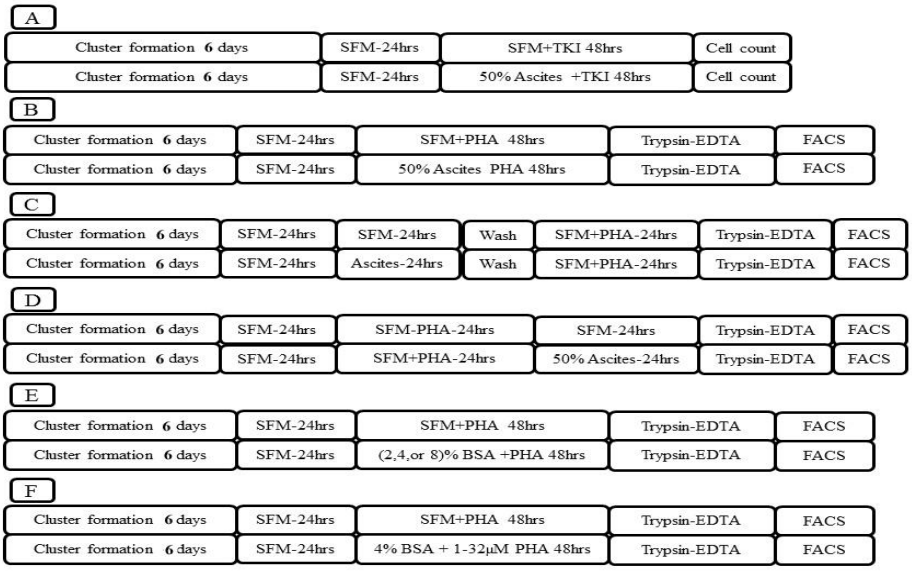


Figure.2.1. Treatment scheme to explore the effect of ascites and BSA on cellular uptake of TKI. OVCAR-5 cellular clusters and SKOV-3 compact aggregates were grown for 6 days in working medium supplemented with 5% FBS, then incubated in serum-free medium (SFM) for 24 hours before exposing them to the following conditions: **(A)** 50% (v/v) ascites with single agent (4 µM canertinib or 2 µM PHA665752), or a combination of 3 µM canertinib and 1 µM PHA665752. Cells were incubated with the ascitic fluid and inhibitors for 48 hours and a cell count was performed to determine the growth. **(B)** Clusters and compact aggregates were exposed to 50% (v/v) ascitic fluids containing 2 µM PHA665752 for 48 hours before trypsinising and analysing via FACS, **(C)** Clusters and compact aggregates were exposed to 50% ascites for 24 hours, and washed with SFM, then incubated with SFM supplemented with 2 µM PHA665752 and followed by incubation for further 24 hours, before trypsinising and analysing via FACS. **(D)** Clusters and compact aggregates were incubated with PHA665752 for 24 hours before the addition of 50% (v/v) ascitic fluids and further incubation for 24 hours before trypsinising and analysing via FACS. **(E)** Clusters and compact aggregates were incubated with SFM supplemented with 2, 4, and 5% (w/v) BSA supplemented with 2 µM PHA665752 for 48 hours before trypsinising and analysing via FACS. **(F)** Clusters and compact aggregates were incubated for 48 hours with SFM supplemented with 4% BSA, which also contain a range of concentrations of PHA (0-32) µM. Cells were trypsinised and analysed via FACS and cell count. All experiments were independently performed at least three times with triplicates.

2.14 Ovarian Cancer Cell Adhesion Assays

In order to investigate cell adhesion in the presence of growth factors and tyrosine kinase inhibitors, cells were cultured as 3D cell clusters and aggregates for 6 days in working medium before further incubation for 24 hours in serum free medium (SFM) (see section 2.2.). Cells were then treated with SFM supplemented with 0.2 or 20 ng/mL growth factor and canertinib or PHA665752 alone or a combination of the two inhibitors for 4 hours before performing the cell adhesion assay. Cells were then transferred onto a collagen-gel matrix and incubated for another 4 hours in a CO_2 incubator at 37°C. After 4 hours of incubation, cellular metabolism was assayed by the Alamar blue dye assay (see section 2.6.), numbers of adherent and non-adherent cells were counted (see section 2.7.) and the expression of proteins of interest were investigated by Western blotting (see section 2.9).

The cell adhesion assay was carried out on the surface of a collagen gel matrix, which was composed of 2 mg/ml collagen 1 and 25% Geltrex (a collagen IV and laminin rich matrix). The plates, coated with collagen, were freshly prepared during the 4 hours of incubation with the inhibitors. To prepare the collagen gel matrix, a total volume of 7 mL of collagen-gel matrix solution was made up as follows: 1.75 mL of 100% Geltrex, for a final concentration of 25% (Life Technologies, Auckland, New Zealand), 2.8 mL acid soluble collagen 1 solution, for a final concentration of 2.5 mg/mL (Life Technologies, Auckland New Zealand), topped up with 2.45 mL base medium. For preparation of 2.8 mL of 2.5 mg/mL acid soluble collagen 1 solution, 2.33 mL of collagen 1 stock solution (3 mg/mL) was added to 280 µL of 1x PBS and 35 µL of 1 N NaOH (for a final concentration of 0.0125 N), topped up with 155 µL MilliQ water. Two hundred microliters were poured into each well of a 24 well plate and incubated at 37 °C for 30 minutes; these were left to cool down at room temperature before adding the cellular clusters and aggregates and incubating at 37 °C for a further 4 hours. Assessment of cellular metabolic activity was carried out using Alamar blue before trypsinisation of the cells and the number of adherent and non-adherent cells was counted.

Immunofluorescent imaging of the receptor tyrosine kinases EGFR and HER-2 as well as the integrin subunits β1 and β4 were performed via staining the cellular clustars and aggregates with specific antibodies for the receptors as described before in section 2.11. Details of antibodies used are detailed in table 2.1.

Experiments exploring the effect of the presence of RGD motif on the cell adhesion in the presence or absence of the inhibitor combination were also performed. Cells were treated for 4 hours as above with the combined inhibitors in the presence of RGDS, an integrin-ECM inhibitor, or RGES (as a control for RGDS inhibitory effects) and also acetate as a vehicle control. Then adhesion to collagen-gel matrix was applied and adherent cells were counted.

2.15 Statistical analysis

Statistical analysis of data was carried out using GraphPad Prism® software (La Jolla, CA, USA). One way and two-way ANOVAs were carried out where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were considered to indicate levels of statistical significance. Prior to analysis percentile and ratiometric values were logarithmically transformed. All data are presented as mean ± SEM. Each experiment was repeated at least three times.

Table. 2.1. Working concentrations of antibodies used for Western Blots and immunofluorescent analysis.

Primary Antibody	Size (kDa)	Company/ serial number	Working dilution	Blocking buffer	Secondary Anti body	Species
EGFR	170	SC-03	1/500	1% BSA	1:10000	Rabbit
p-EGFR	170	SC-101668	1/500	1% BSA	1:10000	Rabbit
HER-2	185	SC-284	1/1000	1% BSA	1:5000	Rabbit
p-HER-2	185	SC-12352-R	1/500	1% BSA	1:10000	Rabbit
c-MET	145	SC-10	1/500	5% Skim milk	1:10000	Rabbit
p-MET	145	SC-101736	1/500	1% BSA	1:10000	Rabbit
ERK1/2	42	SC-94	1/2000	5% Skim milk	1:5000	Rabbit
p-ERK	44	SC-7383	1/500	1% BSA	1:10000	Mouse
Akt	56-63	SC-8312	1/500	1% BSA	1:10000	Rabbit
p-Akt	56-63	SC-101629	1/500	1% BSA	1:10000	Rabbit
GAPDH	37	SC-25778	1/2000	5% Skim milk	1:5000	Rabbit
PCNA	36	SC-25280	1/500	5% Skim milk	1:10000	Mouse
β1	132		1/500	1% BSA	1:10000	Rabbit
β4	200	SC-9090	1/1000	1% BSA	1:5000	Rabbit
Cytokeratin-18 (CK-18)	41-44	SC-5259	1/500	5% Skim milk	1:10000	Mouse
β-Actin (C-4)	42	SC-47778	1/500	5% Skim milk	1:10000	Mouse

*SC Santa Cruz USA

CHAPTER THREE

THE EFFECT OF A COMBINATION OF TWO SMALL MOLECULE TYROSINE KINASE INHIBITORS ON THE GROWTH OF OVARIAN CANCER CELL IN AN *IN VITRO* 3D MODEL

3.1 Introduction

Some of the mutations that underlie cancer are those that lead to the over expression of growth factor receptors. These may affect the signalling pathways that regulate cell growth, migration and survival. EGFR, HER-2 and c-MET are receptor tyrosine kinases suggested to be highly expressed in several types of cancers including ovarian cancer [84, 280, 288, 306, 456, 459-467]. Their over expression is associated with an invasive phenotype and poor prognosis in ovarian cancer patients [305, 342, 344, 459, 466, 468, 469]. Because these receptors are present at low levels in normal cells, inhibitors targeting EGFR, HER-2 and c-MET can potentially inhibit the growth of malignant cells, but have limited adverse effects on normal cells [470]. Thus, an anticancer drug that could inhibit the growth of malignant cells and potentially prevent their spread to other organs may have a significant impact on the improving patient's quality of life. Ovarian cancer cells have been shown to utilise multiple signalling pathways to promote growth and proliferation [467, 471, 472], therefore, targeting of one specific receptor or pathway may have limited effect on growth and proliferation [1]. For instance, a limited effect was observed upon treatment of ovarian tumours with EGFR or PDGFR inhibitors [473, 474]. It has been recognised that combining anticancer drugs that target multiple pathways can be beneficial in cancer treatments [475-477].

A group of anti-cancer drugs collectively termed 'small molecule tyrosine kinase inhibitors' have been extensively investigated. Some of those inhibitors e.g. gefitinib, erlotinib, and lapatinib, which were approved by the FDA, are specific to EGFR and HER-2 and have been found to be beneficial for the treatment of different malignancies including breast cancer, endometrial cancer, colon cancer, head and neck cancer, and non-small cell lung cancer (NSCLC) [215, 241, 242, 259, 462, 478-480] However, in ovarian cancer trials these inhibitors have shown limited positive clinical outcomes as single agents [219, 481]. The abundance of EGFR/HER-2 and c-MET are thought to contribute to resistance to treatments and this may explain the low efficacy of cancer treatments in some types of cancers [482, 483].

EGFR and HER-2 belong to a family of four transmembrane receptors EGFR, HER-2, Her-3 and Her-4. With the exception of HER-2, all other receptors are activated upon binding to the epidermal growth factor (EGF). HER-2 has no known binding ligands; instead it is co-activated by heterodimerisation with other members of the family, especially EGFR [460, 461, 484]. The c-MET proto oncogene is a receptor tyrosine kinase that binds to hepatocyte growth factor (HGF) [333, 483, 485]. EGF and HGF are growth factors known to be elevated in the ascitic fluids of women with advanced stages of ovarian cancer [486, 487]. Activation of EGFR, HER-2 or c-MET receptors subsequently triggers a cascade of downstream signalling processes essential for cell survival, motility, and invasion. Crosstalk between these three receptors may promote cancer progression and increase resistance to therapy [488]. Thus, targeting c-MET in addition to EGFR and HER-2 may impact cell survival.

This chapter will investigate whether targeting two different receptor tyrosine kinases using two different specific inhibitors can enhance the efficacy of the drugs and possibly reduce the growth of ovarian cancer cell lines.

The inhibitors tested were canertinib (CI-1033), an irreversible pan ErbB inhibitor [251, 308, 489, 490], and PHA665752, which is a reversible c-MET inhibitor [254, 255]. Canertinib has shown potent effects against several cancer cell lines targeting EGFR and HER-2. Pre-clinical and clinical trials of canertinib have been conducted against a number of tumours, including breast cancer [221, 491], NSCLC [478] and advanced ovarian cancer [219, 492]. However, canertinib alone showed modest activity in clinical studies with ovarian cancer patients [219]. *In vivo* studies indicated that PHA665752, at low concentrations, has anti-tumour activities [255, 256]. The presence of growth factors in the tumour microenvironment, either naturally occurring or secreted by tumour cells, is essential for cell growth. EGF and HGF, specific ligands for EGFR/HER-2 and c-MET respectively, activate the receptors upon binding and autophosphorylation of the receptors occurs. Activation of these receptors may be useful for studying the efficacy of drugs in advanced ovarian cancer since most ovarian cancer cells are thought to express high levels of these receptors and utilise them for survival [397, 493-495].

3.2 Hypothesis and Aims

The hypothesis of this chapter is that a combination of two receptor tyrosine kinase inhibitors (canertinib and PHA665752) will compromise cell growth, cellular metabolism and tyrosine kinase activity in a 3D cell model of advanced ovarian cancer.

The aim of this chapter is to investigate the effects of canertinib and PHA66572 on clusters and compact aggregates of tumour cell cultures and correlate these with the expression of EGFR, HER-2, c-MET and their downstream signalling molecules.

3.3 Results

3.3.1 Morphology of 3D cell clusters/compact aggregates and expressions of receptors

The first stage in the investigation was to study the morphology of OVCAR-5 cell clusters and SKOV-3 compact aggregates and determine any possible differences between them that may affect their respective responses to treatments. OVCAR-5 and SKOV-3 cells were grown in 12 well plates that were pre-coated with poly-hydroxy ethyl methacrylate (poly-HEMA) polymer to induce cellular aggregation. Cells were incubated for 4 days with culture media and allowed to form 3D clusters and aggregates. Cellular aggregates were then collected and imaged using an inverted light microscope. OVCAR-5 cells formed small clusters (**Figure 3.1 A**) while SKOV-3 cells formed large, dense cellular aggregates (**Figure 3.1 B**).

Next the expression of EGFR, HER-2 and c-MET in both cell lines was investigated. Cells were cultured for 4 days in working media before immunofluorescent labelling and imaging using a fluorescent microscope. OVCAR-5 cells expressed both EGFR and c-MET, however, HER-2 expression was undetectable (**Figure 3.1 C, G** and **E** respectively). In contrast, SKOV-3 compact aggregates stained positive for EGFR, HER-2 and c-MET (**Figure 3.1 D, F,** and **H** respectively). The staining of proteins is located at the cell membrane of cancer cells. These results are consistent with the literature confirming the presence of the receptors in the cell lines under investigation [334, 397, 465, 493].

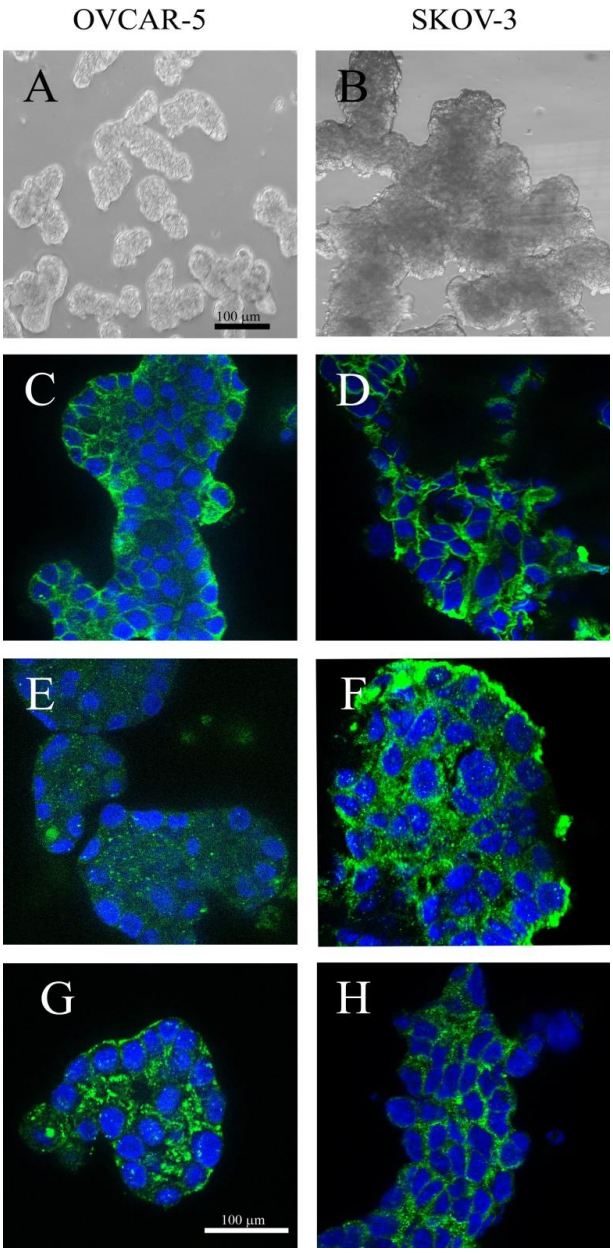


Figure 3.1. Morphology of cellular clusters/ compact aggregates and the expression of EGFR, HER-2 and c-MET (all indicated by green staining). Images shown: (A) OVCAR-5 cellular clusters; (B) SKOV-3 compact aggregates; (C) EGFR in OVCAR-5 and (D) EGFR in SKOV-3; (E) HER-2 in OVCAR-5; (F) HER-2 in SKOV-3; (G) c-MET in OVCAR-5; and (H) c-MET in SKOV-3 clusters and aggregates.

3.3.2 Growth factors induced cell growth, did not affect cell metabolism, and increased expression of proliferating cell antigen (PCNA)

3.3.2.1 Cell growth and cellular metabolism

To study the effect of growth factors on the ovarian cancer cell lines, two concentrations of EGF and HGF were used, a lower concentration that is physiologically relevant (0.2 ng/mL) and a higher concentration (20 ng/mL) matching that used by other workers [278, 435, 496].

Results showed a concentration dependent increase in cell growth of both cell lines with increased growth factor concentration [**Figure 3.2. (A)** OVCAR-5 and **(B)** SKOV-3]. The effect of HGF was more prominent than that of EGF. Using an Alamar blue assay no significant effect on cellular metabolism was observed (**Figure 3.2. (C)** OVCAR-5 and **(D)** SKOV-3).

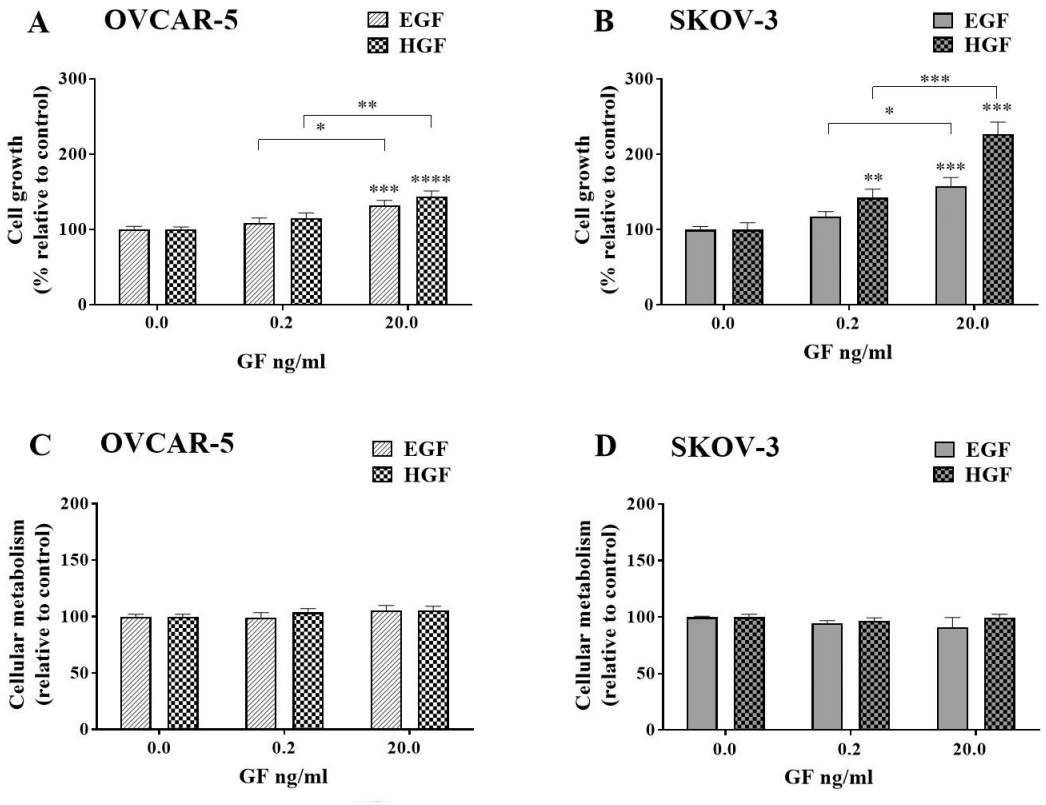


Figure 3.2. The effect of two growth factors on cell growth activity and cellular metabolism of the two ovarian cancer cell lines. Data are shown for OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**), clusters/compact aggregates, treated with 0.2 and 20ng/mL of epidermal growth factor (EGF), hepatocyte growth factor (HGF). Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.2.2 Expression of Proliferating Cell Nuclear Antigen (PCNA) increased in the presence of the growth factors

Once cells are in the proliferative phase, PCNA expression would be expected to increase as it surrounds the DNA and acts as a platform for recruitment of proteins involved in DNA replication, DNA repair and chromatin remodelling. Since an increase in cell growth in the presence of growth factors was established in this chapter, an investigation of the expression of PCNA with the growth factors was carried out. Overall, PCNA expression was increased with increased growth factor concentrations (EGF or HGF), in both OVCAR-5 (**Figure 3.3 A** and **E**) and SKOV-3 (**Figure 3.3 B** and **F**). A densitometry index obtained by comparing the PCNA band intensity to the band intensity of the house keeping protein, GAPDH, also indicated increased levels of PCNA in both OVCAR-5 (**Figure 3.3 C** and **G**) and SKOV-3 (**Figure 3.3 D** and **H**) cells. Protein concentrations in growth factor treated OVCAR-5 clusters and SKOV-3 compact aggregates were significantly higher than in untreated controls (see appendix).

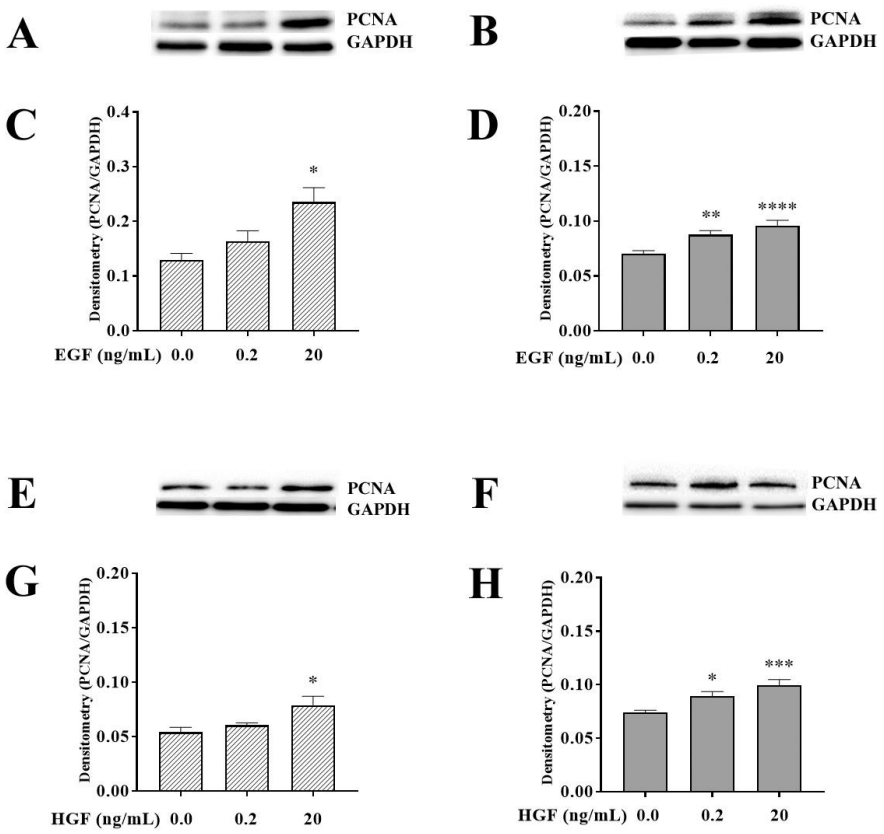


Figure 3.3. The effect of growth factors on the expression of PCNA in ovarian cancer cell lines. Western blotting and densitometry index of PCNA data are shown for clusters/compact aggregates of OVCAR-5 treated with EGF (**A**, **C**) and HGF (**E**, **G**), and SKOV-3 cells treated with EGF (**B**, **D**) and HGF (**F**, **H**). Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.3 The concentration dependence of canertinib and PHA665752 in the presence of fetal bovine serum (FBS) or growth factors

In order to investigate the response of the cells to the inhibitor treatments, first their response upon activation with FBS was studied. FBS contains an array of growth factors, amino acids, lipids, cytokines, and chemokines that are essential for cell growth and thus may contribute to the activation of the receptors and thus a cellular response or resistance if any, to the inhibitors.

3.3.3.1 Response to canertinib in the presence of 5%FBS

Cell numbers of both cell lines were significantly reduced by canertinib in a concentration dependent manner, as shown in **Figure 3.4 (A)** for OVCAR-5 and **(B)** for SKOV-3 cell lines respectively. There were no observed changes in cellular metabolism for OVCAR-5 (**Figure 3.4 C**), while SKOV-3 showed a significant reduction in metabolism (**Figure 3.4 D**). These results may suggest that SKOV-3 has an overall better response to canertinib than OVCAR-5, probably due to the higher expression of EGFR and HER-2.

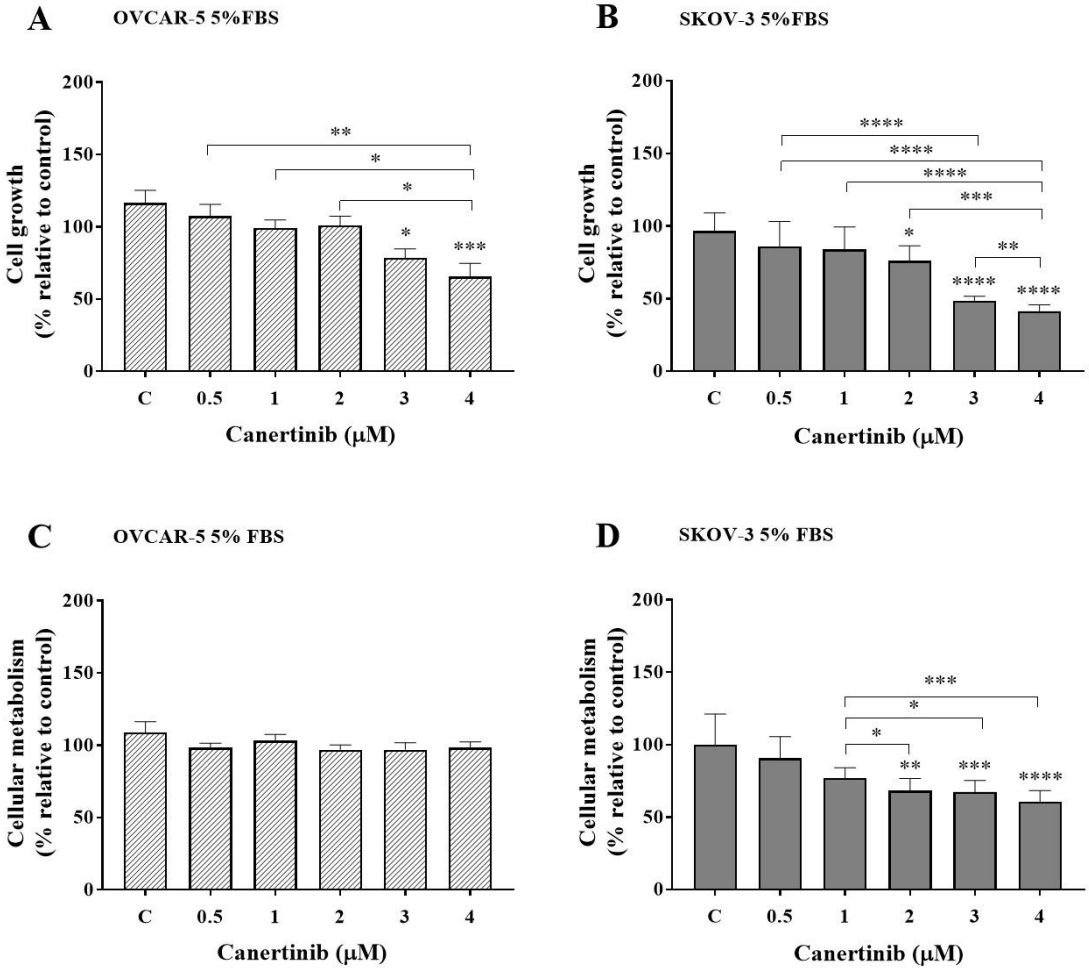


Figure 3.4. The effect of canertinib on cell growth and cellular metabolism of OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**) ovarian cancer cell lines. Clusters and compact aggregates were treated with canertinib at concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 μM in the presence of 5% fetal bovine serum (FBS). Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.3.2 Response to PHA665752 (PHA) in the presence of 5%FBS

OVCAR-5 cell clusters exhibited a significant concentration dependent decrease in cell growth with PHA665752 (**Figure 3.5 A**). This may be due to the high expression of c-MET in OVCAR-5 cells and may suggest that OVCAR-5 cells utilise c-MET as a primary growth receptor. However, SKOV-3 compact aggregates showed a more modest response to the c-MET inhibitor, with a significant decrease in cell growth only at concentrations of 2 µM or above (**Figure 3.5 B**). This may suggest that SKOV-3 compact aggregates may utilise EGFR/HER-2 as primary growth receptors. Only 2 µM PHA665752 significantly reduced cellular metabolism in both cell lines (**Figure 3.5 C** and **D**).

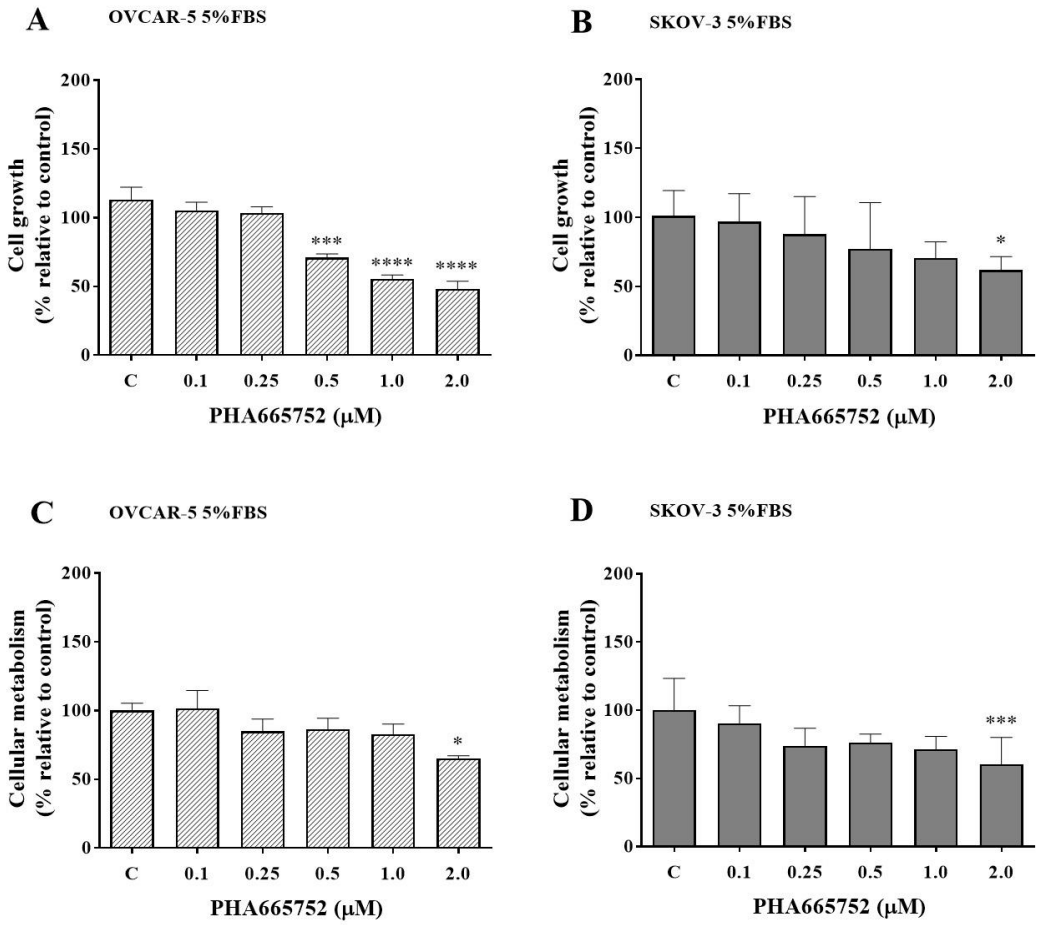


Figure 3.5. The effect of PHA665752 on cell growth and cellular metabolism of the ovarian cancer cell lines OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**). Clusters and compact aggregates were treated with PHA665752 at concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.0 μM in the presence of 5% FBS. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.3.3 The effect of growth factors (GF) on the efficacy of cantertinib

The tumour microenvironment contains different growth factors and other substances that may affect the drug efficacy and cellular response [497-499]. The focus of this study was to assess the effect of the growth factors, EGF and HGF on drug efficacy. As mentioned earlier, growth factors play a pivotal role in the growth of cancer cells. The presence of EGF or HGF is essential for activation of EGFR/HER-2 or c-MET, respectively. Upon binding to the growth factors, the receptors dimerise and auto phosphorylation of the tyrosine residues occurs at the intracellular portion of tyrosine kinase domain. This process would in turn induce phosphorylation of intracellular signal transduction proteins that signal cell growth and proliferation. To investigate the cellular response in the presence of growth factors, OVCAR-5 clusters and SKOV-3 aggregates were treated with different concentrations of the inhibitors in the presence of 20 ng/mL EGF and HGF. Canertinib elicited a concentration dependent inhibition of cell growth in both EGF treated OVCAR-5 (**Figure 3.6 A**) and SKOV-3 (**Figure 3.6 B**) cells. Similar results were obtained when HGF was present, as shown in **Figure 3.6 C** and **D** for OVCAR-5 and SKOV-3 respectively.

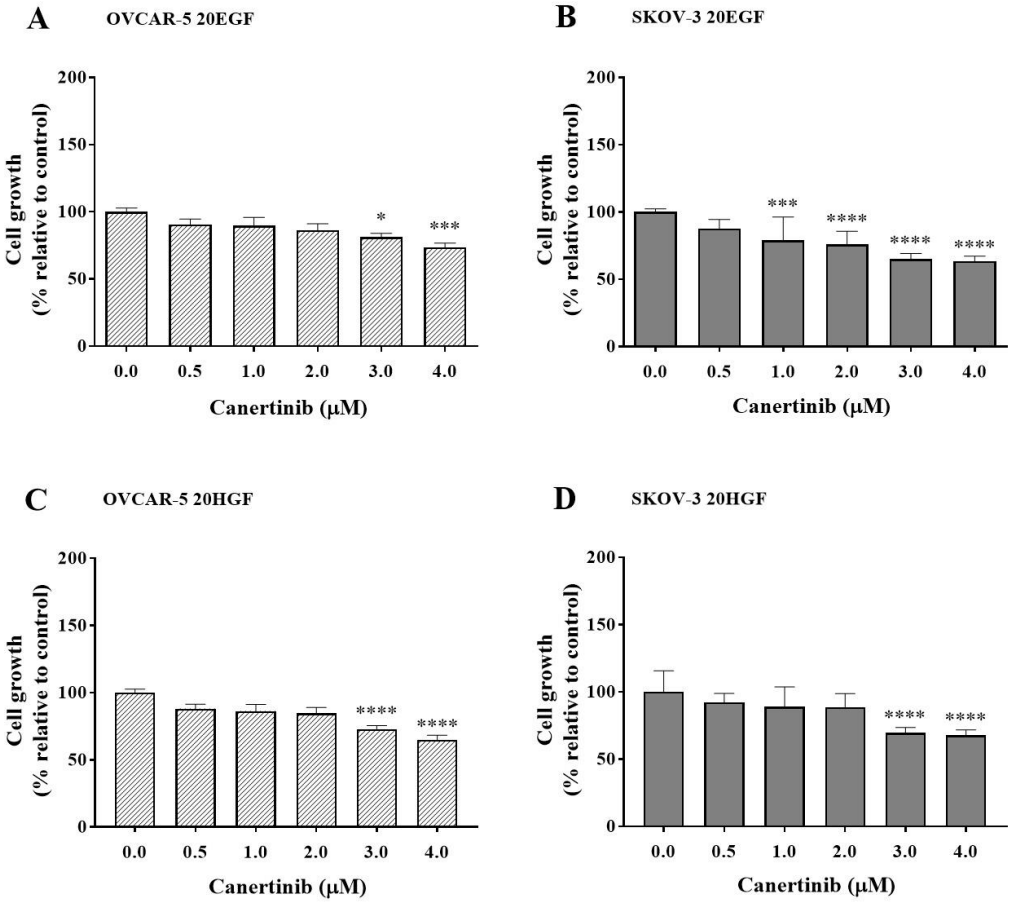


Figure 3.6. The effect of canertinib on cell growth of ovarian cancer cell lines OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**). Cell clusters and compact aggregates were treated with canertinib at concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 μM in the presence of 20 ng/mL of EGF or HGF. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.3.4 The effect of growth factors (GF) on the efficacy of PHA665752

Both cell lines showed a concentration dependent decrease in cell growth, after treatment with PHA665752, in the presence of EGF [**Figure 3.7 A** (OVCAR-5) and **B** (SKOV-3)]. Similar results were obtained with HGF for both OVCAR-5 (**Figure 3.7 C**) and SKOV-3 (**Figure 3.7 D**) cells.

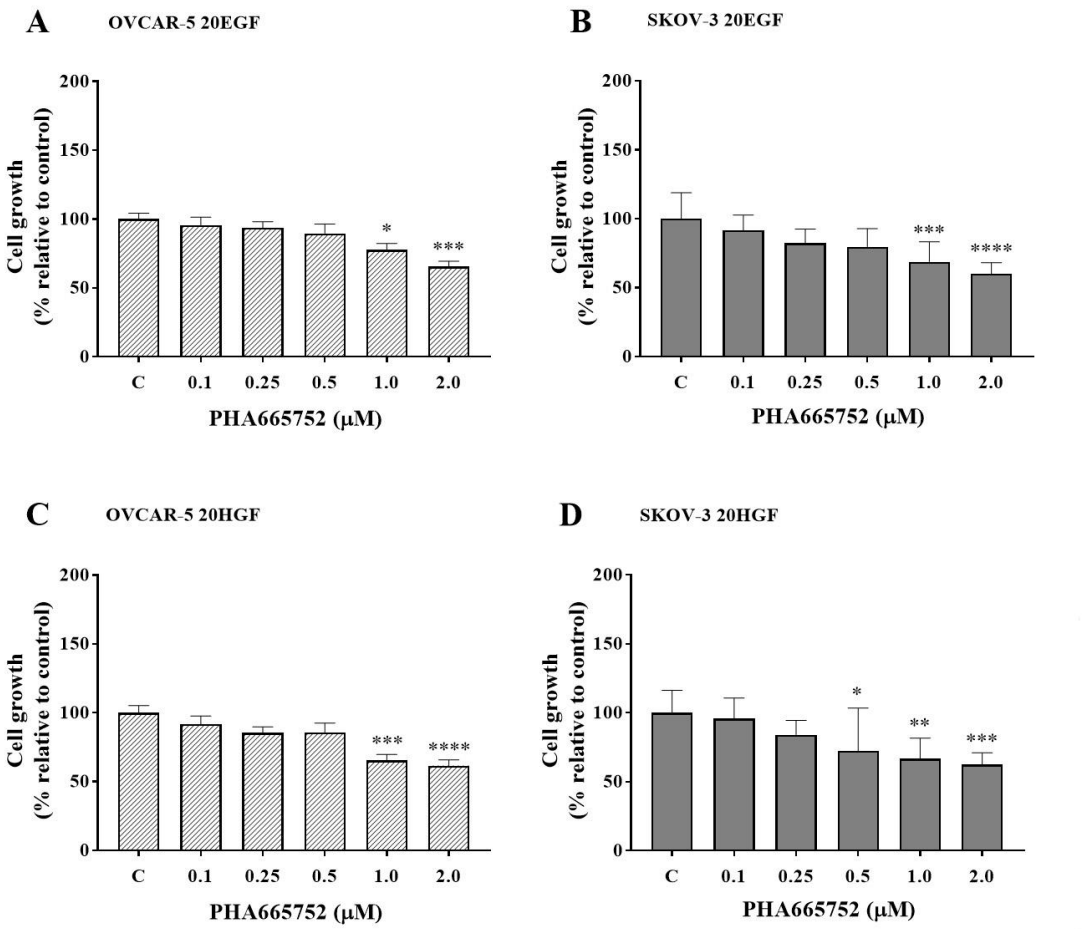


Figure 3.7. The effect of PHA665752 on cell growth of two ovarian cancer cell lines OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**). Cell clusters and compact aggregates were treated with PHA665752 (PHA), 0.1, 0.25, 0.5, 1.0, 2.0 μM in the presence of 20 ng/mL EGF or HGF respectively. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.3.5 The effect of a combination of two growth factors on the efficacy of canertinib

As demonstrated in the previous sections, catertinib concentrations above of 0.5 µM affected the growth of OVCAR-5 cell clusters and SKOV-3 compact cellular aggregates. In order to demonstrate the effect of the presence of growth factors on the cellular response to the treatments, OVCAR-5 clusters and SKOV-3 compact aggregates were exposed to 4 µM canertinib alone in a serum free medium (SFM), i.e., no growth factors or FBS). In the absence of growth factors, there was minimal effect of canertinib on cell growth of both OVCAR-5 clusters (**Figure 3.8 A**) and SKOV-3 aggregates (**Figure 3.8 B**). This may suggest that the presence of growth factors is mandatory for both ovarian cancer cell lines responding to the inhibitors. Therefore, OVCAR-5 clusters and SKOV-3 compact aggregates were stimulated with a mixture of EGF and HGF (henceforth this combination will be referred to as GF) and the effects of the inhibitors were investigated. The concentrations of the growth factor mixture used in this study were 0.2 ng/mL of GF, a physiologically relevant level for patients with advanced ovarian cancers [500], and 20 ng/mL of GF, a concentration that has been used in other studies [421, 465].

Canertinib significantly inhibited cell growth of OVCAR-5 cellular clusters in the presence of 0.2 or 20 ng/mL of GF, in a concentration dependent fashion, (**Figure 3.8 C**). This supports the suggestion that activation with growth factors is required to trigger the cellular response to the treatments. The inhibiter was more potent with SKOV-3 cell aggregates, having a greater inhibitory effect on cell growth (**Figure 3.8 D**). Cellular metabolism in SKOV-3 compact aggregates (**Figure 3.8 F**) was also decreased in a concentration dependent manner, regardless of the concentration of the growth factors. However, reduction in cellular metabolism of OVCAR-5 (**Figure 3.8 E**) was only statistically significant with 20 ng/mL of GF but not with 0.2 ng/mL of GF.

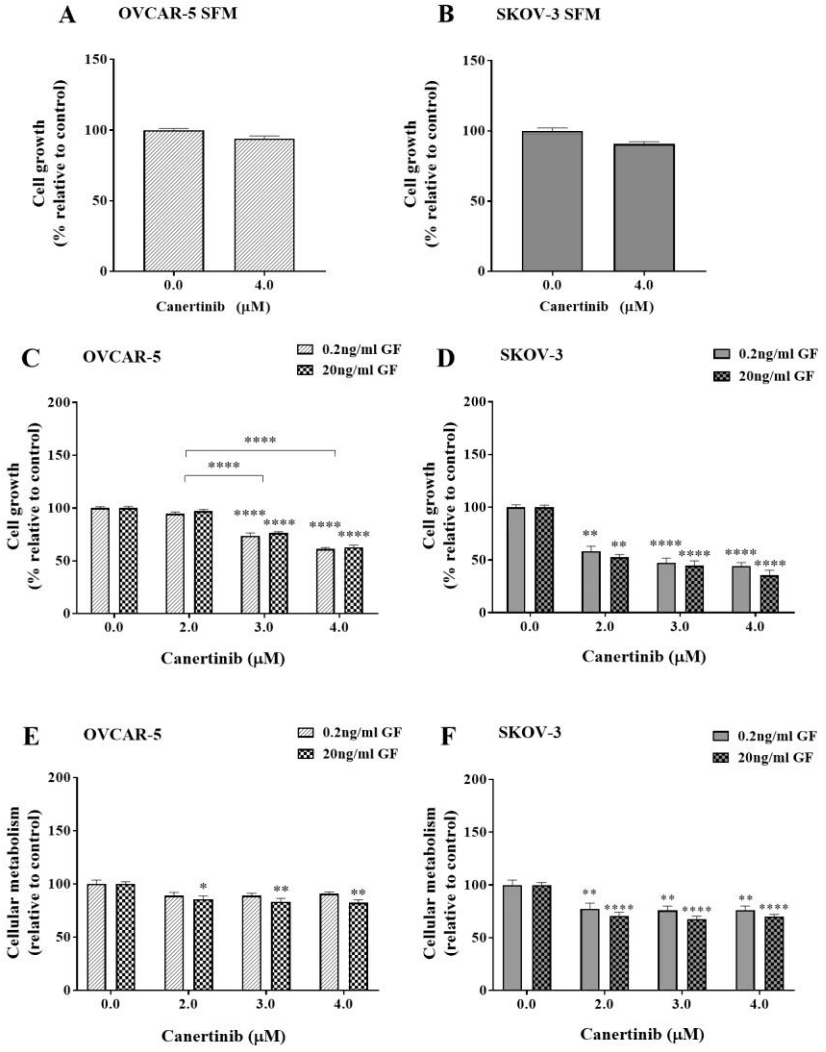


Figure 3.8. The effect of canertinib on cell growth and metabolism of the two ovarian cancer cell lines OVCAR-5 and SKOV-3. Cell clusters of OVCAR-5 (**A**) and SKOV-3 compact aggregates (**B**) were cultured in serum free medium (SFM), and exposed to 4 µM canertinib. OVCAR-5 (**C** and **E**), and SKOV-3 (**D** and **F**) treated with canertinib at concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 µM in the presence of 0.2 or 20 ng/mL GF. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.3.6 The effect of a combination of two growth factors on the efficacy of PHA665752

In the absence of growth factors, PHA665752 exhibited limited efficacy on both OVCAR-5 cell clusters (**Figure 3.9 A**), and SKOV-3 compact aggregates (**Figure 3.9 B**). However, in the presence of growth factors, PHA665752 inhibited the growth of OVCAR-5 clusters in a concentration dependent manner, irrespective of the concentration of growth factors (**Figure 3.9 C**). This effect was less prominent in SKOV-3 cellular aggregates, and only consistent at the lower concentration of growth factors (**Figure 3.9 D**). This may suggest that due the high expressions of EGFR and HER-2 on SKOV-3 compact aggregates, the cells may have been utilising them as an alternative pathway to maintain survival. In contrast, a significant concentration dependent reduction of cellular metabolism in OVCAR-5 (**Figure 3.9 E**) cells was found with 20 ng/mL of GF. However, when 0.2ng/mL GF was present, only higher concentrations of the inhibitor were effective. Cellular metabolism of SKOV-3 cells was not significantly reduced with increasing concentrations (**Figure 3.9 F**).

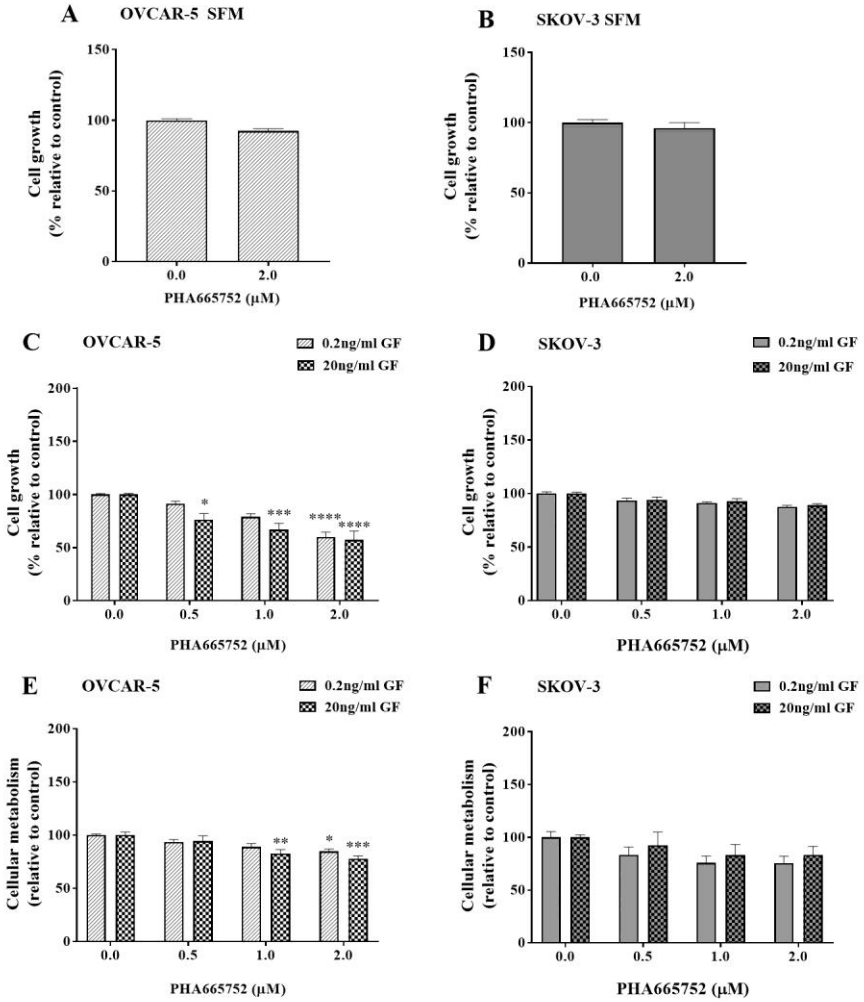


Figure 3.9. The effect of PHA665752 on cell growth and metabolism of the two ovarian cancer cell lines OVCAR-5 and SKOV-3. Cell clusters of OVCAR-5 (**A**) and compact aggregates of SKOV-3 (**B**), were cultured in serum free medium (SFM) and exposed to 2 µM PHA665752. Clusters of OVCAR-5 (**D** and **F**), were treated with PHA665752, 0.1, 0.25, 0.5, 1.0, 2.0 µM in the presence of 20 ng/mL GF. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.4 The most effective concentrations for inhibitor combination experiments

In order to investigate the optimal concentration for the inhibitor combination treatments a series of experiments were performed to identify their lowest effective concentrations. Cells were exposed to different combinations of the inhibitors, for 48 hours in the presence of 20 ng/mL of GF and cell growth and metabolic activity were analysed. Results show a significant decrease in the growth of OVCAR-5 clusters (**Figure 3.10 A**) and SKOV-3 compact aggregates (**Figure 3.10 B**), with a combination of 3.0 µM canertinib and 1.0 µM PHA665752. A marked decrease in cellular metabolism was observed for both cell lines at the same concentrations of the inhibitor combination (**Figure 3.10 C** and **D**).

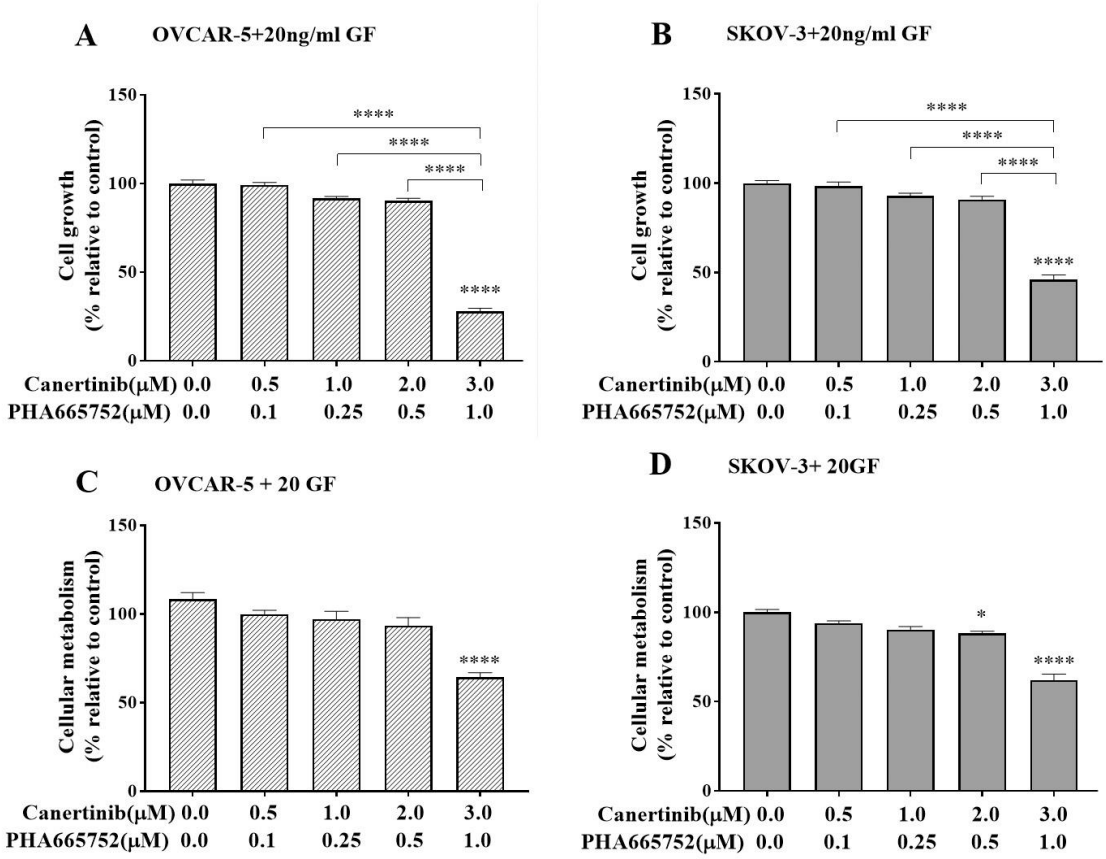


Figure 3.10. The effect of different combinations of canertinib and PHA665752 on cell growth and metabolism of the two ovarian cancer cell lines OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**). Cell clusters and compact aggregates were treated with selective combinations of canertinib and PHA665752, (0.5, 0.1), (1.0, 0.25), (2.0, 0.5), and (3.0, 1.0) μM respectively, in the presence of 20 ng/mL GF. Data are expressed as means ± S.E.M (n=4). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.5 The effect of a combination of canertinib and PHA on cell growth and metabolism in the presence of 5%FBS

While this study is focused on the effect of combining the two inhibitors in the presence of growth factors, the effect of the combination in the presence of FBS is also of interest. FBS can influence a number of additional growth promoting agents that may affect the cellular response to the treatments. In the presence of FBS, a significant reduction in cell numbers for both cell lines was observed with the inhibitors applied individually or in combination (**Figure 3.11 A** and **B** for OVCAR-5 and SKOV-3 respectively). There were no changes in cellular metabolism of OVCAR-5 (**Figure 3.11 C**), and the only slightly significant reduction in cellular metabolism for SKOV-3 was noted with the combination treatment (**Figure 3.11 D**).

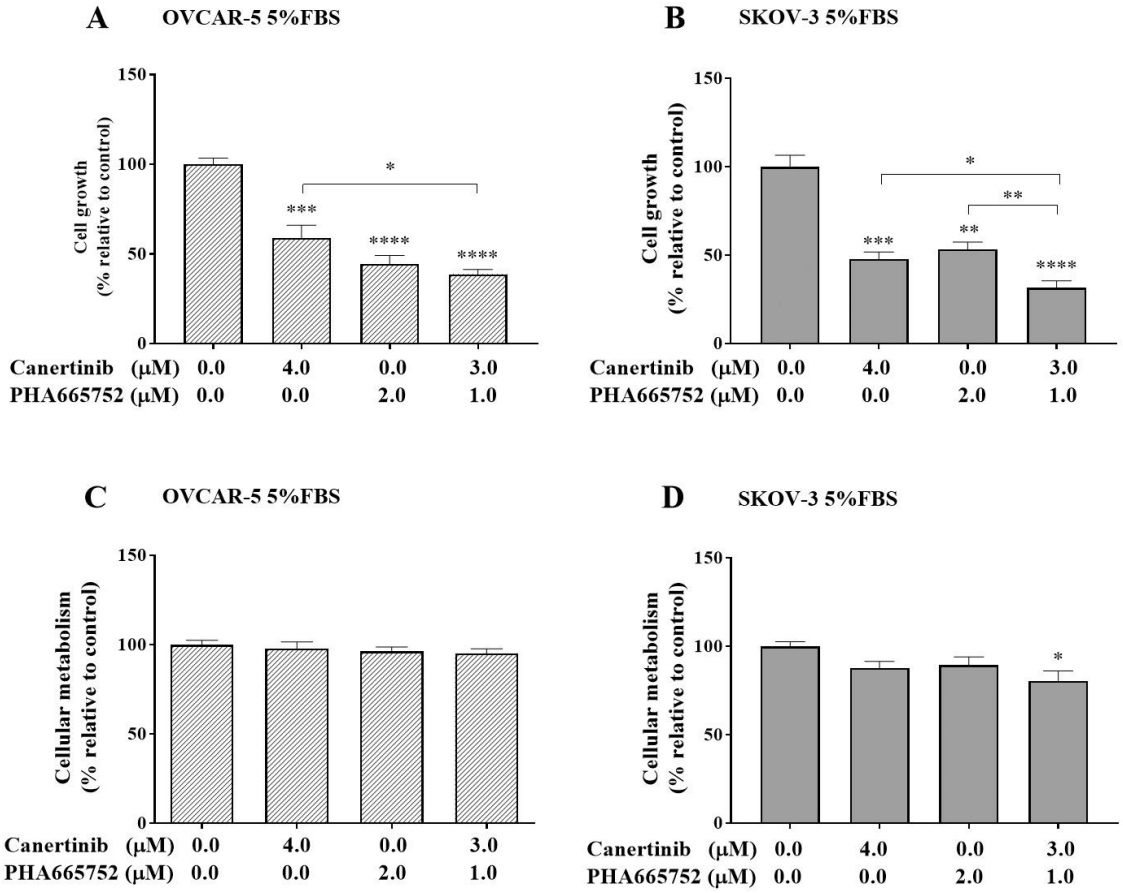


Figure 3.11. The effect of a combination of canertinib and PHA665752 on cell growth and metabolism of the ovarian cancer cell lines OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**). Cell clusters and compact aggregates were treated with canertinib or PHA665752 alone or in combination in the presence of 5%FBS. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.6 Effect of the combination of canertinib and PHA665752 on cell growth, proliferation and metabolism in the presence of 0.2 or 20 ng/mL GF.

The data presented above indicate that the lowest effective combination of the inhibitors was 3.0 μM canertinib and 1.0 μM PHA665752 and that the growth factor concentration of 0.2 ng/mL (close to physiological levels for women with advanced ovarian cancer) was sufficient for activation of the receptors and triggering a cellular response to the treatments. However, for comparison with results reported by other workers, a concentration of 20 ng/mL of GF was also tested. The level of cell proliferation was determined by using proliferating cell nuclear antigen (PCNA) as a marker. In the presence of 0.2 ng/mL GF, cell proliferation was reduced and the expression of PCNA, as measured using Western blotting and densitometry readings relative to the housekeeping protein GAPDH, was reduced especially with the combination of inhibitors [**Figure 3.12 A** and **B** for OVCAR-5 and SKOV-3 respectively]. Cell growth of both OVCAR-5 clusters and SKOV-3 aggregates was reduced when each of the inhibitors was used separately; and further inhibition of the cell growth occurred when both inhibitors, canertinib and PHA665752, were used in combination (**Figure 3.12 C** and **D** respectively). Similar patterns of inhibition were observed in both cell lines when 20 ng/mL of GF was used (**Figure 3.13**.).

There are two possible scenarios as to why increasing the growth factor concentrations did not enhance the cellular response to the inhibitors; firstly, the growth factors are such potent cytokines that the minimal concentration will activate the receptors and the excess growth factor concentrations are left in the cell culture medium.

Cellular metabolism of OVCAR-5 clusters was reduced with PHA665752 alone and in combination, but not with canertinib, with both 0.2 and 20 ng/mL GF (**Figure 3.14 A** and **C**) respectively. However, SKOV-3 compact aggregates showed a significant decrease when canertinib and PHA665752 were used separately or in combination, with both growth factor conditions (**Figure 3.14 B** and **D**). Protein levels, in treated samples, as would be predicted, were significantly lower than the controls.

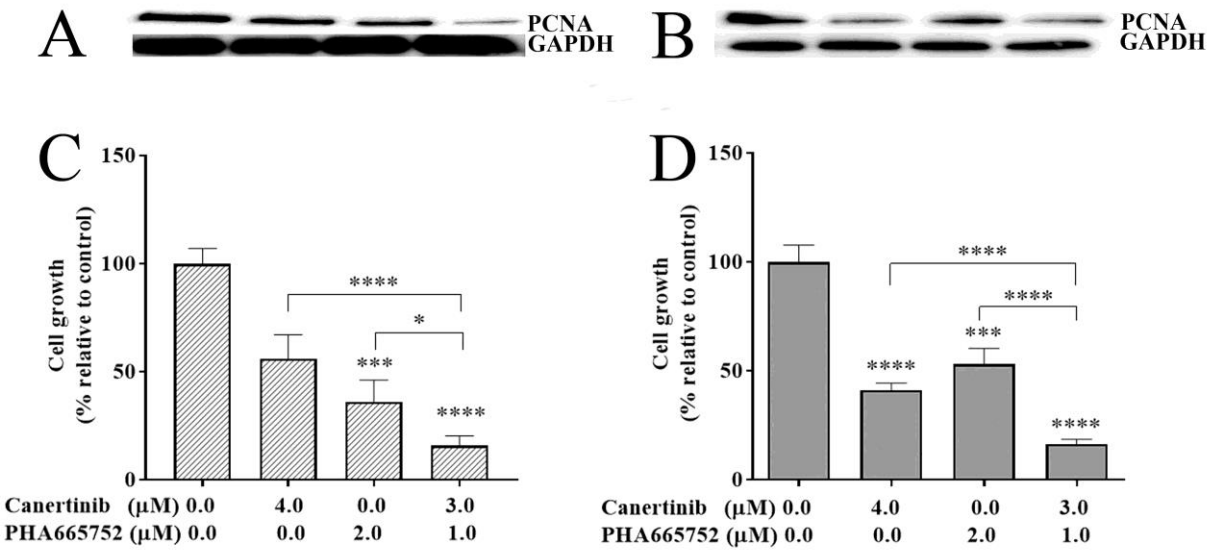


Figure 3.12. Western blot showing the expression of PCNA and a reference protein (GAPDH) in ovarian cancer cellular clusters/compact aggregates of (**A**) OVCAR-5 and (**B**) SKOV-3 treated with each inhibitor separately or in combination, in the presence of 0.2 ng/mL of GF. Cell growth of OVCAR-5 cells (**C**) and SKOV-3 (**D**) for the same conditions. Cell growth data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

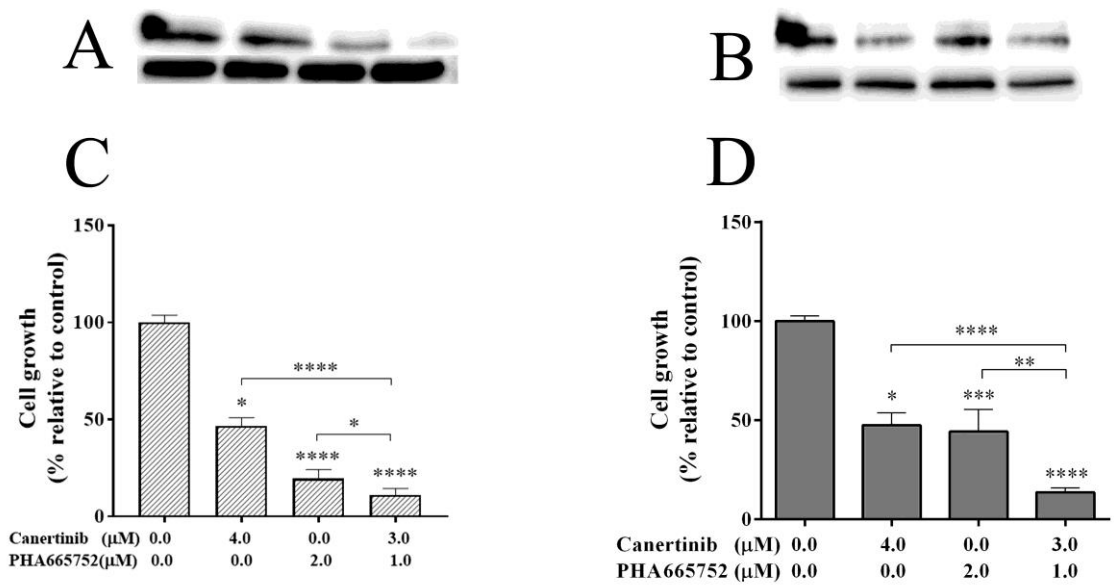


Figure 3.13. Western blot showing the expression of PCNA and a reference protein (GAPDH) in ovarian cancer cellular clusters/compact aggregates, OVCAR-5 (**A**) and SKOV-3 (**B**), treated with each inhibitor separately and in combination, in the presence of 20 ng/mL of GF. Cell growth of OVCAR-5 cells (**C**) and SKOV-3 (**D**) for the same conditions. Cell growth data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

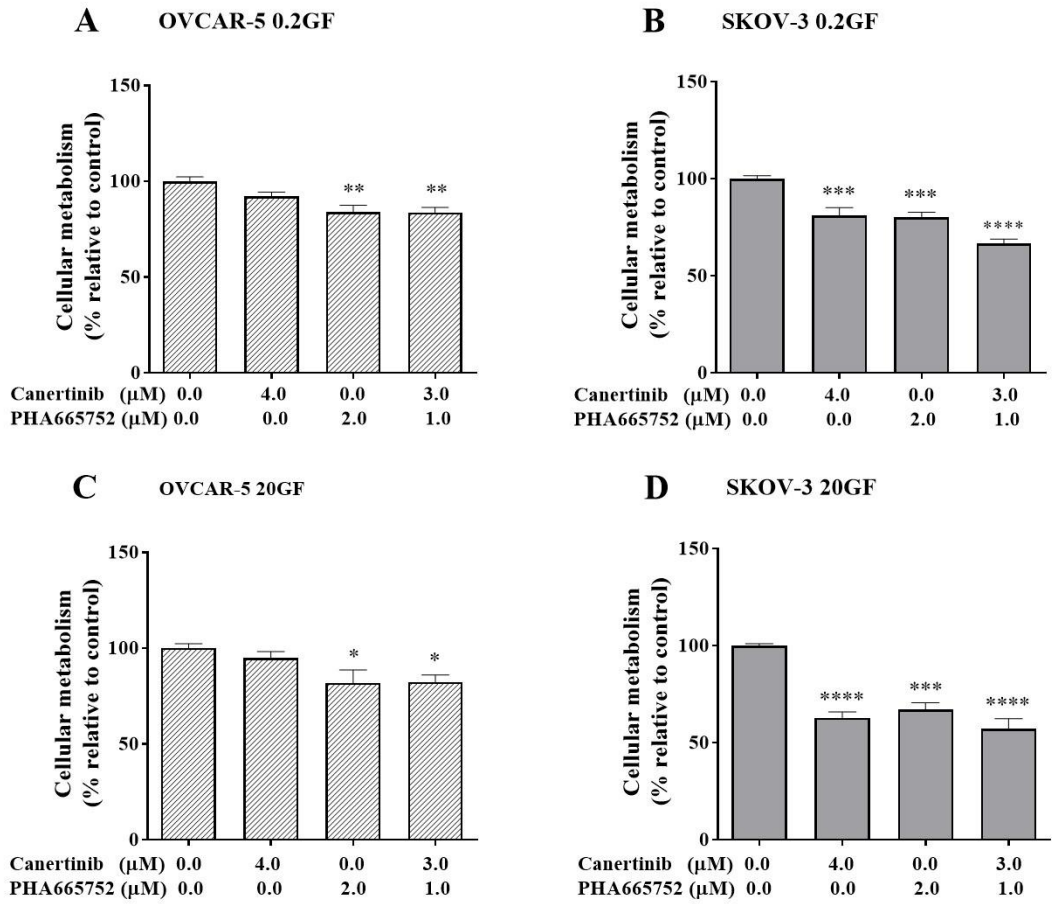


Figure 3.14. The effect of a combination of canertinib and PHA665752 on cellular metabolism of the ovarian cancer cell lines OVCAR-5 (**A**, **C**) and SKOV-3 (**B**, **D**). Cell clusters and compact aggregates were treated with a single concentration and a combination of canertinib and PHA665752 in the presence of 0.2 or 20 ng/mL of GF. Cellular metabolism data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.7 The effect of the combination of canertinib and PHA665752 on the receptors and downstream signalling molecules

The previous results suggest that cell growth and cellular metabolism are markedly reduced upon treatment with canertinib and PHA separately, and a further reduction was noted when the cells were treated with the combination of the inhibitors. There was also a reduction in cellular metabolism upon exposure to the inhibitors. In the following section, the effect of the inhibitors on total expression and phosphorylation of the target receptors EGFR, HER-2 and c-MET as well as the total expression and phosphorylation of the downstream signalling molecules Akt and ERK is investigated.

3.3.7.1 The effect of inhibitors on the receptors and signalling molecules in OVCAR-5 cellular clusters

In the presence of 0.2 ng/mL of GF both canertinib and PHA665752 reduced the levels of p-EGFR when they were applied individually or in combination (**Figure 3.15 A, B**).

In contrast, total expression of EGFR was only slightly reduced when the cells were treated with canertinib alone (**Figure 3.15 C**). Treatment with PHA665752 alone significantly reduced the total expression of c-MET and levels of phosphorylated p-MET and this was further reduced when the cells were treated with the combination of the inhibitors (**Figure 3.15 D, E**). Total expression of Akt and phosphorylation was reduced when the cells were treated with the combination (**Figure 3.15 F, G**). There were no significant changes in total expression of ERK, but treatment of the cells with the inhibitors individually reduced p-ERK. A further reduction was noted when they were combined (**Figure 3.15 H, I**). The expression of HER-2 was undetectable in OVCAR-5 clusters. Similar results were observed with 20 ng/mL of GF (**Figure 3.16**).

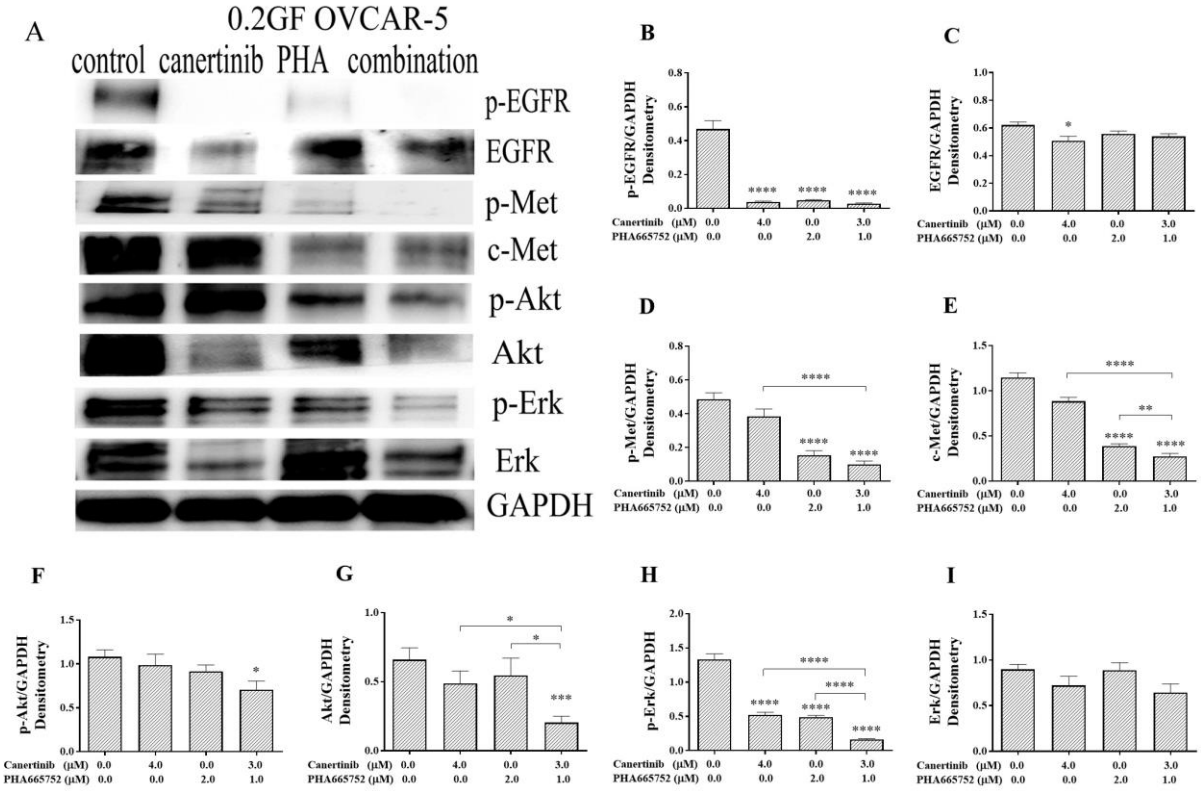


Figure 3.15. Western blotting and densitometry, showing the effect of a combination of canertinib and PHA665752 (PHA), in OVCAR-5 clusters, on total expression and phosphorylation of EGFR, Her-2, c-Met, Akt and Erk, with 0.2 ng/mL of GF. Densitometry values are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

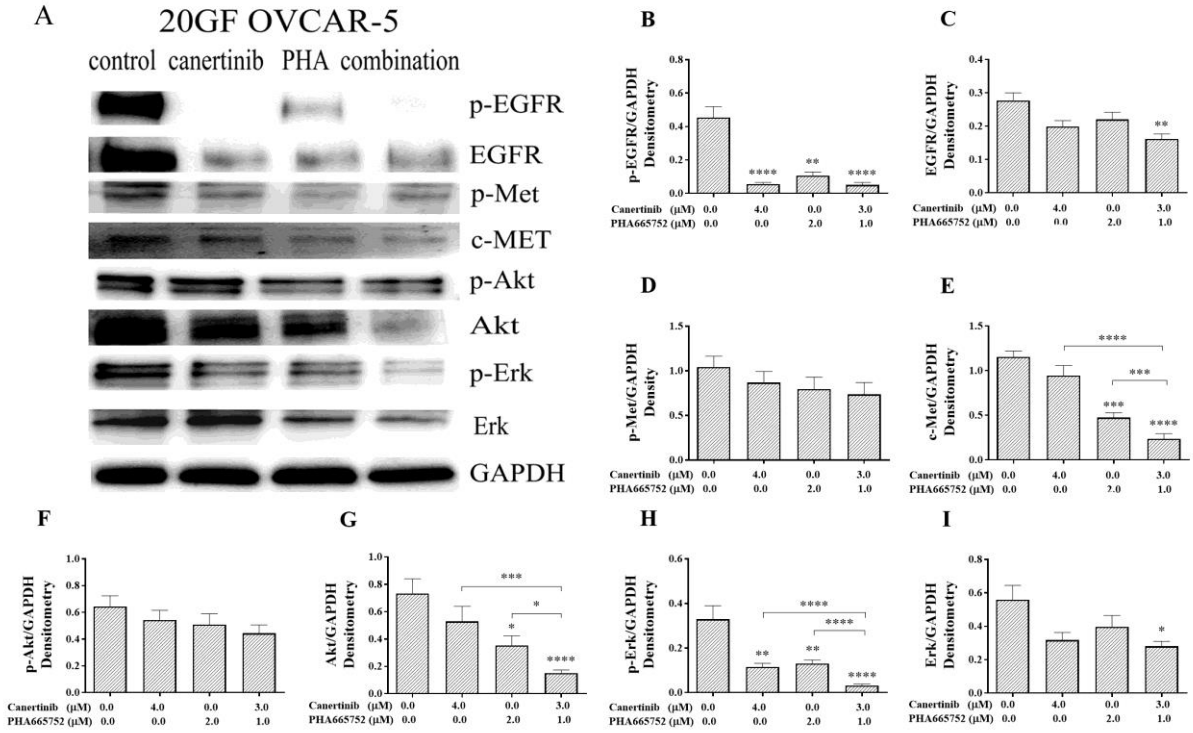


Figure 3.16. Western blotting and densitometry, showing the effect of a combination of canertinib and PHA665752 (PHA), in OVCAR-5 clusters, on total expression and phosphorylation of EGFR, Her-2, c-Met, Akt and Erk, with 20 ng/mL of GF. Densitometry values are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.7.2 Effect of the inhibitors on the receptors and signalling molecules in SKOV-3 compact aggregates

The effects of the inhibitors were tested in presence of 0.2 ng/mL of the GF. Canertinib alone or in combination with PHA665752 reduced the expression of p-EGFR in SKOV-3 compact aggregates in the presence of 0.2 ng/mL of GF (**Figure 3.17 A, B**). However, there was no significant reduction when PHA665752 was used alone indicating that the reduction was mainly due to canertinib inhibition (**Figure 3.17 A, B**). Total expression of EGFR, was also significantly reduced with the use of canertinib alone or in combination, but not with PHA665752 alone (**Figures 3.17 C**). A reduction in the phosphorylation and total expression of HER-2 was observed with canertinib alone or in combination (**Figure 3.17 D, E**). Canertinib alone and in combination, reduced p-MET, and both inhibitors, both individually and in combination significantly reduced total c-MET (**Figure 3.17 F, G**). A reduction in Akt and p-Akt was also notable with the combination (**Figure 3.17 H, I**). Canertinib alone reduced the total expression and phosphorylation of ERK, with a further reduction with the combination (**Figure 3.17 J, K**). Similar trends were obtained with 20 ng/mL of GF, but the reduction of c-MET and p-MET was more prominent and PHA665752 was more potent when applied individually (**Figure 3.18**).

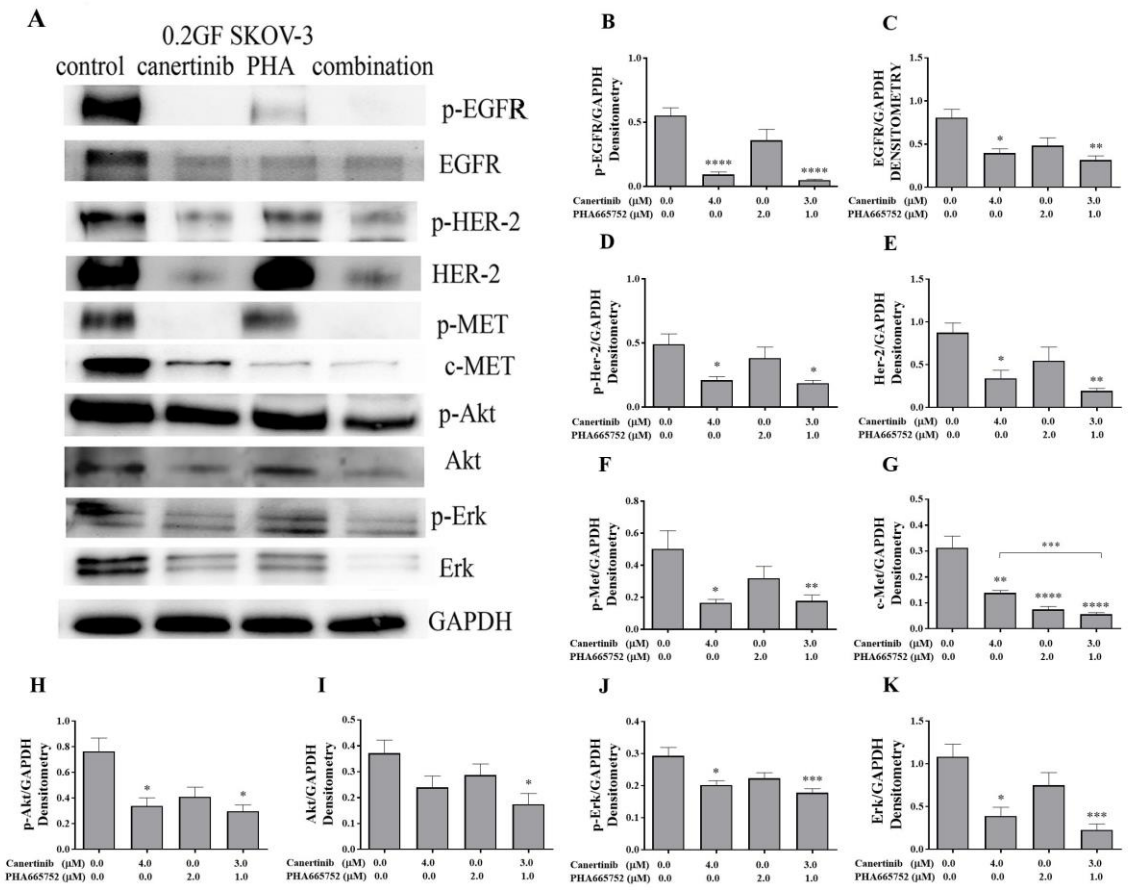


Figure 3.17. Western blotting and densitometry index showing the effect of a combination of canertinib and PHA665752 on total expression and phosphorylation of EGFR, HER-2, c-MET, Akt and ERK of SKOV-3 compact aggregates, in the presence of 0.2 ng/mL of GF. Densitometry values are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

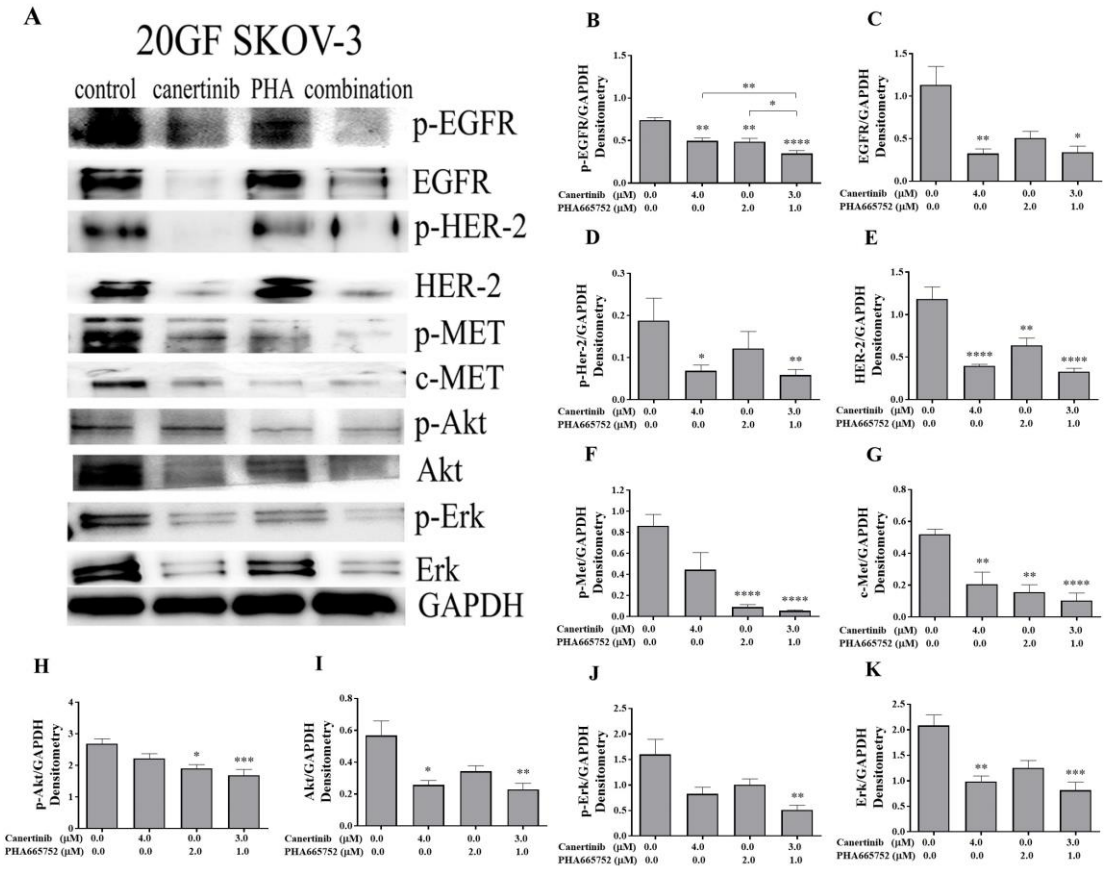


Figure 3.18. Western blotting and densitometry showing the effect of a combination of canertinib and PHA665752 on total expression and phosphorylation of EGFR, HER-2, c-MET, Akt and ERK of SKOV-3 compact aggregates, with 20 ng/mL of GF. Densitometry values are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.3.8 The effect of the combination of canertinib and PHA665752 on Vascular Endothelial Growth Factor (VEGF) secretion from OVCAR-5 and SKOV-3 ovarian cancer cell lines

VEGF secretion is the first step in the process of angiogenesis and it is a characteristic of malignant cells. VEGF stimulates microvascular hyper permeability before and during angiogenesis leading to tumour vascularisation and spread [436]. The presence of growth factors and cytokines in the surrounding microenvironment, of ovarian cancer cells, induces the secretion of VEGF, which acts as a signal to promote cell migration and metastasis. High expressions of certain oncogenes, such as EGFR /HER-2, has been shown to upregulate VEGF signalling and this in turn will contribute to resistance to EGFR inhibitors [501]. To investigate the effect of the combination of canertinib and PHA665752 on VEGF secretion, the cellular clusters and compact aggregates were exposed to the inhibitors and a VEGF ELISA was performed to estimate VEGF secretion.

In the presence of 0.2 ng/mL of GF, OVCAR-5 clusters showed a significant decrease in VEGF with the combination treatment but no marked reduction with the single inhibitors (**Figure 3.19 A**). However, with 20 ng/mL of GF, a reduction in VEGF was observed with both, single inhibitors and with the combination (**Figures 3.19 B**). SKOV-3 compact aggregates showed a decrease in VEGF secretion with single treatment of canertinib and with the combination in both 0.2 and 20 ng/mL of GF (**Figure 3.19 C and D**).

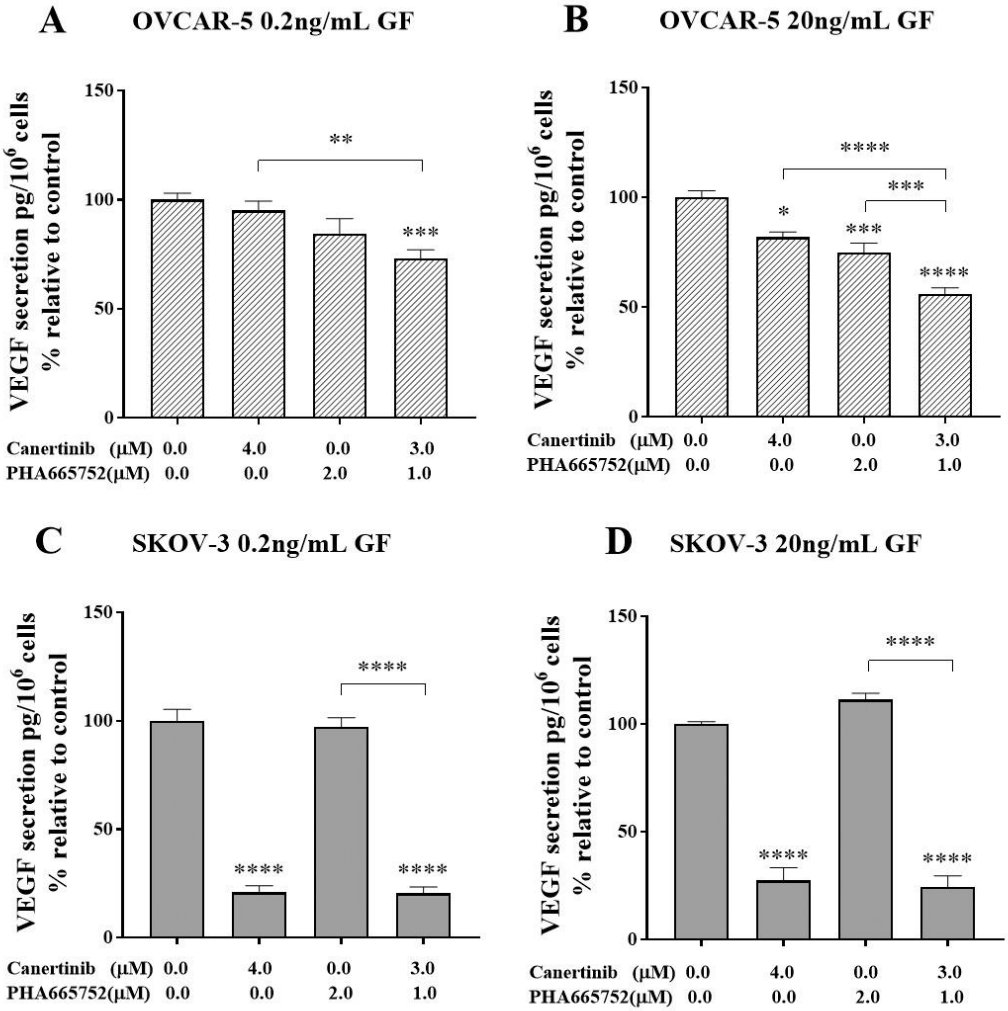


Figure 3.19. The effect of a combination of canertinib and PHA665752 on VEGF secretion in the two ovarian cancer cell lines OVCAR-5 (**A**, **B**) and SKOV-3 (**C**, **D**). Data show VEGF secretion from cell clusters and compact aggregates treated with canertinib and PHA665752 alone or in combination in the presence of 0.2 or 20 ng/mL of GF. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

3.4 Discussion

The effects of the combination of inhibitors targeting the receptor tyrosine kinases EGFR, HER-2 and c-MET were investigated in two ovarian cancer cell lines, OVCAR-5 and SKOV-3. During intraperitoneal dissemination, ovarian tumour cells shed into the abdominal cavity and survive as single cells or free floating cellular clusters and aggregates in the ascitic fluid [7, 156]. Therefore, in order to closely mimic the tumour microenvironment, cells were cultured as floating cellular clusters and compact aggregates. Such 3D cultures have a better potential to replicate the cellular heterogeneity of malignant ovarian cancer cell clusters and compact aggregates that are floating in the ascitic fluid [502-505]. As such, the 3D cell model may reflect a closer representation of the potential response to the drug combination *in vivo* [502, 503].

3D cell culture models have been developed to mimic the *in vivo* environment and investigate the progression and resistance mechanisms of several types of cancer cells including those of the ovary [506, 507]. Muranen and colleagues studied the effect of small molecule inhibitors of the PI3K/mTOR pathway in a 3D cluster model of ovarian and breast cancer, a pathway that is frequently mutated in epithelial cancers [508]. They found that tumour cells that attached to the matrix in the core of the spheroid were resistance to the PI3K/mTOR inhibitors most likely through the upregulation of pro-survival proteins. In contrast, proliferation of 2D monolayers of cells was inhibited although apoptosis was not observed. It was suggested that the PI3K/mTOR pathway was not inhibited in the inner part of the spheroids while 2D cultures and outer cells exhibited strong survival [508]. This may explain the limited effects of PI3K/mTOR inhibitors in previous studies where 2D cell monolayer may exhibit loss of cellular polarity, and ECM-adhesion induced survival. In another study Lee and colleagues investigated 31 ovarian cancer cell lines. They compared the molecular and biological properties of ovarian cancer cells grown in 2D and 3D models with xenografts and ovarian tumours. They used poly-HEMA polymer coating of culture plates to establish the 3D spheroids. This study revealed a distinct alteration in protein expressions, proliferation rates and chemo-sensitivity in the 3D models which closer mimicked the xenograft in vivo characteristics than the 2D monolayer cultures [509].

With respect to cell morphology, my findings were similar to previous reports that indicated that OVCAR-5 cells form small clusters and SKOV-3 cells form larger cellular aggregates [509, 510]. Also consistent with other studies, the results showed that OVCAR-5 clusters express high levels of EGFR and c-MET, but have low HER-2 expression. SKOV-3 compact aggregates express high levels of all three receptors [397, 511, 512].

Growth factors such as EGF and HGF are involved in phenotypical modulation of ovarian cancer cell behaviour and thus are likely to play a pivotal role in metastatic cell migration [278, 513-515]. They are associated with phenotypic modulation of ovarian cancer cells and aggressive tumours and this correlates with a poorer prognosis [516, 517]. High levels of EGF and EGFR have been detected in tumours, ascitic fluid and urine of patients with advanced ovarian cancer [518, 519]. Binding of EGF to EGFR will trigger a cascade of downstream signal transduction pathways including Ras-MAPK, PI3K-Akt, and STAT [520]. Moreover, the overexpression of epidermal growth factor receptors in advanced ovarian cancer cells and the association with rapid growth and disease spread have been reported [521, 522].

HGF is responsible for activation of growth pathways that lead to invasive disease [470, 523-525] and aberrant activation of HGF pathway induces invasive cell growth phenotype in cancer [526]. HGF stimulation of the PI3K-Akt signalling pathway has been correlated with increased motility and invasion of ovarian cancer cells [523]. The high levels of HGF found in the serum of women with a pelvic mass (>2 standard deviations above serum levels of women with benign tumours) is a sign of malignant manifestation and of a poor prognosis [527]. Additionally, the high levels of HGF in ascitic fluids from advanced ovarian cancer patients (reported levels of between 4.1-16 mg/mL) [487], are linked to resistance to targeted therapies, aggressive disease and poor prognosis in patients with epithelial ovarian cancers [337, 486, 487]. Moreover, high HGF levels in tumour cells are often associated with a reduced progression-free survival for the patient with advanced epithelial ovarian cancer [389]. HGF is a specific ligand for the c-MET receptor tyrosine kinase. The c-MET proto-oncogene has been found to be overexpressed in 11% of advanced ovarian cancer patients [334]. Recently, Davies et al., have reported that 96% of malignant tissue obtained from ovarian cancer patients stains positive for the c-MET receptor [288]. Zhou et al. suggested that ovarian cancer cells are consistently exposed to both EGF and HGF in the tumour

microenvironment and they both contribute to aggressive metastasis by activation of overlapping intracellular signalling pathways [526]. They found that EGF and HGF could increase cell growth in a concentration dependent manner within the range of 6.25-2400 pM (approximately 0.0375-14 ng/ml). Similar findings are presented in this study In contrast to these results, some studies report only a minor increase in cell proliferation in SKOV-3 monolayer cells treated with HGF for 3-5 days [528].

Cancer cells control their own metabolism according to their biosynthetic needs. Zhao et al. in reviewing literature suggest that energy production by many (but not all) cancer cells occurs mostly via aerobic glycolysis. The abnormally increased aerobic glycolysis, as well as other metabolic pathways in cancer cells has been associated with drug resistance. Zhao et al conclude that agents that reduce cellular metabolism may provide effective treatments [529]. Hence, the effects of the inhibitors on cellular metabolism were investigated.

While the results in this chapter report an increase in cell growth and total protein concentration, there were no changes in cellular metabolism in the presence of the growth factors. This controversial outcome of cellular metabolism finding may be due to the fact that the experiments were carried out on 3D cell cultures. The 3D model is a complicated system where the cells are aggregated in floating clusters. These clusters and aggregates differ in surface area and the number of cells that form each cluster. Thus, the number of cells that are located on the outer surface of the cluster and hence that are not exposed to Alamar blue may not be equal even within the same sample and the bigger the cluster, the greater the number of cells that are exposed. This suggests that the assay of cellular metabolism may be influenced more by the surface area of clusters rather than the concentration of growth factors. Additionally microscopic observation revealed subpopulations of single floating cells that were not attached to the clusters. This is consistent with the fact that ovarian cancer cells survive in the ascitic fluid either as single cells or as clusters and aggregates [156]. These numbers of these single cells differed between tested samples. Thus the relative amounts of these single floating cells may affect the results of the cellular metabolism assay. Given this, it would be interesting to investigate cellular metabolism in single cell suspensions of the samples of the same treatments via trypsinisation of the clusters prior to the metabolism assay.

The effects of the small molecule inhibitors canertinib, which is an irreversible pan EGFR family inhibitor, and PHA665752, which is a reversible c-MET specific inhibitor, in the presence of EGF and HGF were evaluated. The selected inhibitor concentrations used were consistent with those in a previous study of hepatocellular carcinoma in mice [530]. Small molecule inhibitors of EGFR such as Gefitinib (ZD1839, Iressa) and Erlotinib (OSI-774, Tarceva) are reversible and have been approved by the FDA in the US for treatment of NSCLC [531]. However, many of these drugs do not induce a sustained clinical outcome with EGFR positive cancers [532, 533]. Canertinib is an inhibitor that irreversibly binds to the ATP pocket of the intracellular domain of the EFGR family of receptors. Unlike reversible tyrosine kinase inhibitors, an irreversible inhibitor may exert prolonged clinical activity against the target receptor. The advantage of the irreversible inhibitor is the permanent binding to the receptor, which can be translated into more sustained inhibition and the need for potentially lower doses [218]. A second favourable feature of canertinib is the rapid pan inhibition of all four members of the ErbB family of receptor tyrosine kinase [534]. There is thus good rationale for using an irreversible inhibitor to ensure a sustained inhibition of the receptors and their downstream signalling molecules [535].

The efficacy of canertinib has been investigated in several types of cancers including breast, NSCLC, and ovarian cancer. In breast cancer, both *in vitro* and *in vivo* xenograft studies have demonstrated the anti-tumour effects of canertinib [248]. However, a phase II trial in platinum refractory ovarian cancer was indefinite, showing stable disease but no measurable response [219]. Adverse effects were seen in patients treated with canertinib, there include nausea, diarrhoea and skin rash [536].

The results presented here indicate that there is a concentration dependent inhibition of growth and metabolism of SKOV-3 compact aggregates treated with canertinib. The growth of OVCAR-5 clusters was inhibited less and there was no change in cellular metabolism. The higher effectiveness of canertinib on SKOV-3 compact aggregates may be due to their aberrant expression of EGFR and HER-2, compared to OVCAR-5 clusters that express less EGFR and have undetectable levels of HER-2 [537]. The low response of OVCAR-5 towards canertinib may be explained by the fact these cells show a low expression of HER-2 and have been shown to have frequent mutations in *BRAF* and *KRAS* [92, 98, 107]. Studies on NSCLC

have shown that cells with high activation mutations in the KRAS pathway are resistant to tyrosine kinase inhibitors and this has been used to explain a poor prognosis with EGFR tyrosine kinase inhibitors [538-540].

Several other studies have shown the inhibitory effect of canertinib against different types of cancers. Irwin et al., [541] showed that canertinib can inhibit the growth of acute lymphoblastic leukaemia cells at concentrations of 0.1-5 μM [541]. The responsiveness of cancer cells to canertinib as a single agent was also observed in HER-2 positive tumours from breast cancer patients [309]. Other studies have shown the inhibitory effects of canertinib on cell proliferation of malignant melanoma cells [542] and breast cancer [246, 248].

PHA665752 is a reversible inhibitor of c-MET receptor tyrosine kinase. PHA665752 when used singly inhibited the growth of OVCAR-5 clusters and SKOV-3 compact aggregates in a concentration dependent manner. However, the effect on cellular metabolism was limited. In comparison with OVCAR-5 clusters, SKOV-3 compact aggregates were less responsive to the c-MET inhibitor. It is possible that the high expression of HER-2 receptors in SKOV-3 may enhance the cells ability to resist the c-MET inhibitor.

Studies have previously suggested that cancer cells may utilise an alternative pathway for growth once the EGFR/HER2 pathway is blocked. Therefore, it can be speculated that when c-MET is blocked, cells that retain high expressions of HER-2 may utilise it as an alternative route. This co-activation of multiple RTKs in cancer cells confers resistance to single-agent therapy. However, this resistance can easily be overcome by simultaneously inhibiting several RTKs with rationally chosen drug combinations [543].

Many other studies have shown the effectiveness of PHA665752. Experiments on lung adenocarcinoma cells with *KRAS* gene mutations have shown that PHA665752 can inhibit growth, proliferation and metastasis [346]. In an *in vitro* study, Ma et al., showed a concentration dependent inhibition of growth of BaF3.TPR-MET cells upon exposure to PHA665752 [544] and Christensen et al., demonstrated that PHA665752, at concentrations of between 0.1-5.0 μM, elicited concentration dependent inhibition of gastric, pancreatic, and lung-carcinoma cell lines [256]. Other studies, in NSCLC and gastric cancers, showed a greater inhibitory effect on growth/metabolism across a range of concentration (0.01-10μM)

It has been suggested that ovarian cancer cells may have the ability to activate multiple receptors for growth, proliferation and metastasis [241, 334, 338]. This co-activation of multiple receptor tyrosine kinases may explain the relatively limited effectiveness of single drug treatments targeting only one of these receptors in these cells [536, 545]. Therefore, there is a rationale for targeting multiple receptors to improve the efficacy of the drugs. Consistent with this, the combination of canertinib and PHA665752 at the lowest effective concentrations for each inhibitor, (which had been previously determined in the concentration dependent experiments with the single inhibitor treatments), compromised cell growth and cellular metabolism more effectively than the single inhibitor alone. Western blot analyses suggest that the combination may modulate the expression of the target receptors and/ or the phosphorylation of their downstream signalling molecules. This is consistent with other studies indicating the inhibitory properties of canertinib on the phosphorylation of EGFR and HER-2 receptor tyrosine kinases in malignant melanoma cells [542].

These results suggest that blocking multiple growth receptors may compromise cell survival in advanced ovarian cancer cell cultures and are consistent with studies on other cancer types. A study of NSCLC and gastric cancer cell lines, by Engelman and colleagues showed that combining PHA665752 with gefitinib reduced cell proliferation more effectively than the single treatments alone [482]. Also Tang et al., reported a greater impact of combined inhibition of both EGFR and HER-2 with reduced growth of EGFR-mutation mediated erlotinib-resistant lung cancer cells [546]. Additionally, Crosswell et al, established that a combination of PHA665752 with rosiglitazone, significantly compromised growth of neuroblastoma cell line [254].

A combination of small molecule inhibitors or monoclonal antibodies with cytotoxic drugs has also been explored in clinical trials for ovarian cancer treatment. The combination of cetuximab, a monoclonal antibody against EGFR, and paclitaxel or carboplatin was effective as a primary therapy for ovarian cancer, but unfortunately this does not ensure a progression-free survival [547]. Also a limited clinical benefit was observed with the combination of lapatinib with topotecan for the treatment of platinum-resistant ovarian cancer [548]. With respect to other cancer types, an *in vivo* animal model of head and neck carcinoma study by Chun et al., [259] demonstrated that a combination of gemcitabine and gefitinib reduced cell

growth and phosphorylation of EGFR as well as levels of the downstream molecule ERK in a synergistic manner [259]. Moreover, a combination of capecitabine with the HER-2 antibody, trastuzumab, or with lapatinib, exhibited clinical potential in the treatment of breast cancer patients, although this was accompanied by an increase in undesirable side effects [549, 550].

As aforementioned, EGF and HGF are potent cytokines that are implemented in the activation of cell growth and proliferation via the upregulation of growth markers including PCNA [496, 551, 552]. This is consistent with the results presented in this thesis that demonstrates the concentration dependent increase in the expression of PCNA in the presence of EGF. There was also a marginal increase in PCNA expression with increasing concentrations of HGF. The downregulation in PCNA expression was observed in both cell lines with single inhibitors and combination. The down regulation of PCNA is consistent with Mayer and colleagues who indicated a decrease in PCNA expression upon growth inhibition [553].

Vascular endothelial growth factor, VEGF, is a key driver of cell proliferation, survival and migration. VEGF receptors are highly expressed in the majority of solid tumours including endocrine, brain, NSCLC, gastrointestinal, breast, kidney, bladder, endometrium and ovarian tumours [554-560]. It plays a pivotal role in the development of normal and pathological ovarian biology, including cancers [432, 437, 473, 561]. Ovarian cancer is characterised by abundantly vascularised tumours and is dependent on VEGF secretion for angiogenesis [562]. VEGF is typically linked to more aggressive tumours and a poor prognosis [153, 563] with high levels of VEGF in malignant ascites from advanced ovarian cancer patients [433]. Studies suggest that blocking VEGF secretion can reduce ascites formation and tumour burden [437, 561, 564]. In this study, canertinib and PHA665752 inhibited VEGF secretion when applied singly and in combination. This is consistent with other studies suggesting that tyrosine kinase inhibitors can inhibit VEGF secretion and compromise vascularisation processes [153, 565].

Functional interactions between VEGF and EGFR have been demonstrated in gefitinib and cetuximab resistant colon cancer cells [566]. Cancer cells that are resistant to EGFR inhibitors are found to express high levels of VEGF, which also correlates with aggressive tumours and

angiogenesis *in vivo* [567]. This correlation is an indicator of intracellular crosstalk between the signalling pathways and poses a promising target for anti-cancer treatments. The tumour environment may be hypoxic and this is regulated by hypoxia-inducible transcription factors (HIFs) by binding to the VEGF promoter to trigger transcription [568]. In context, another transcriptional activation of HIF includes upregulation of EGFR and EGFR signalling [569]. The presence of high levels of VEGF in ovarian cancer malignant ascites is mediated by several factors including EGF and transforming growth factor-β (TGFβ). Furthermore, many EGFR inhibitors including cetuximab and panitumumab and small molecule tyrosine kinase inhibitors such as gefitinib, erlotinib and lapatinib have been shown shown inhibitory actions against VEGF in both *in vitro* and *in vivo* conditions [570]. Thus, angiogenesis is induced by the production of tumour derived VEFG which is stimulated by upregulation of EGFR receptors and activation of downstream signalling molecules [570].

In summary this chapter describes an *in vitro* investigation of the effect of combining two inhibitors of three receptor tyrosine kinases on two ovarian cancer cell lines that form clusters and compact aggregates in a 3D cell culture model. The combination of canertinib and PHA665752 compromised cell growth, cellular metabolism, the expression of receptors, the expression of downstream signalling molecules, and VEGF secretion. The efficacies of the inhibitors under these experimental conditions are of interest but further study on more cell lines is needed before any clinical relevance can be inferred.

CHAPTER FOUR

THE EFFECT OF ASCITIC FLUID ON THE EFFECTIVENESS OF TYROSINE KINASE INHIBITORS

4.1 Introduction

During the progression of ovarian cancer, tumour cells undergo phenotypic differentiation and attain motility, subsequently shedding off from the ovaries. Cells then migrate into the abdominal cavity where they survive as single cells and cellular aggregates [33, 157]. Once the malignant cells seed on the surface of the peritoneum they grow and secrete cytokines and growth factors which, in turn, trigger the leakage of the vascular network underneath the peritoneal wall. This may result in the influx of fluids into the abdominal cavity, a process that is referred to medically as ascites [158, 571]. Malignant ascites is the abnormal accumulation of plural fluid in the abdominal cavity due to tumours and is an indicator of disease spread and a poor prognosis with a reduced 5-year survival rate [161, 165, 176, 572, 573]. It is found in almost 37% of women with advanced ovarian cancers [165, 176, 574] but at a lower percentage with malignant tumours from other organs [575-577].

Malignant ascites comprise a combination of cellular and acellular components [156, 168]. Cellular components include the tumour cells, which are present as both single cells and aggregates [578] along with stromal components that include adipocytes, adipose tissue-derived stem cells, bone marrow-derived stromal cells, fibroblasts, inflammatory cells, endothelial cells, and immune cells [579, 580]. The acellular components of ascites include cytokines, growth factors, chemokines, extracellular matrix components as well as bioactive lipids. All of these can affect the cancer cell behaviour and disease progression [168, 581-584]. Pro-tumorigenic cytokines such as IL-6, IL-8, IL-10 and VEGF are found in significantly increased levels in ovarian cancer ascites compared to plasma. Other cytokines including IL-2, IL-5, and IL-7 are found at lower levels. Collectively, these factors are part of a pro-inflammatory and immunosuppressive microenvironment [585] which plays a role in tumour progression. IL-6 and IL-8, for example are oncogenic stimuli, and are found highly expressed in ovarian cancer ascites and have been shown to be promoters of epithelial-mesenchymal transition where cells regain their invasive properties and angiogenesis [586-588]. VEGF has also been implicated as a promoter of ascites formation and in the metastasis of ovarian cancer cells [153, 589].

Proteomic studies have revealed the presence of up to 2000 proteins in ascitic fluid from ovarian cancer patients [590, 591]. Other components present at high levels in ascites from ovarian cancer patients include RNA-binding proteins, exosomes (cell-derived vesicles that are present in most eukaryotic fluids), low molecular weight metabolites, lysophosphatidic acid (LPA), cholesterol and fatty acids [590].

Ascitic fluids are thought to influence the effect of anti-tumour drugs. The significance of the surrounding microenvironment in the development of cancer is being increasingly acknowledged and it seems to play an essential role in mediating and sustaining the hallmarks of cancer [22, 592]. Cellular aggregates in the ascitic fluid are more likely to exhibit chemo resistance and enhanced malignant potential [158, 571, 593].

As described earlier in this thesis small molecule tyrosine kinase inhibitors (TKIs) are hydrophobic compounds that inhibit receptor tyrosine kinases (RTKs). These inhibitors are promising tools in the development of anti-cancer drugs [594, 595]. In the previous chapter, it was demonstrated that by blocking multiple receptor tyrosine kinases with a combination of two TKIs, canertinib, a pan inhibitor of EGFR family and PHA665752, an inhibitor of c-MET, the growth of clusters and compact aggregates of the OVCAR-5 and SKOV-3 ovarian cancer cell lines can be compromised. The combination of these two inhibitors further reduced the phosphorylation of EGFR, HER-2 and c-MET receptors and downstream signalling proteins p-Akt and p-ERK [596]. However, these effects may not occur in the *in vivo* tumour microenvironment due to the presence of growth promoting substances in the surrounding ascitic fluid that may influence the cellular response to the inhibitors.

In view of the above, this chapter will investigate the efficacy of the two inhibitors on ovarian cancer cell clusters and compact aggregates, in the presence of ascitic fluids from advanced ovarian cancer patients. Such an environment may represent a more accurate representation of the microenvironment for the ovarian cancer cells in the body. This chapter will also investigate the factors underlining any difference in cellular response to the tyrosine kinase inhibitors in this microenvironment.

4.2 Hypothesis

Ascitic fluid from ovarian cancer patients will modulate the behaviour of ovarian cancer cell lines OVCAR-5 and SKOV-3 and their response to treatments with TKIs.

Ascitic fluid will modulate the uptake of TKIs by ovarian cancer cells via binding of albumin to TKIs.

4.3 Aims of the study

1. To determine the effect of malignant ascites on ovarian cancer cell growth and metabolism.

2. To investigate the efficacy of two small molecule tyrosine kinase inhibitors, canertinib and PHA665752, in the presence of ascites from advanced ovarian cancer patients.

3. To investigate the mechanisms by which ascites affect the efficacy of the drugs

4.4 Materials and Methods

Materials and methods are as described in Chapter-2 sections 12 and 13.

4.5 Results

4.5.1 Inhibitor effects on cell morphology in the presence of ascitic fluid.

Ovarian cancer cells can disseminate into the peritoneal cavity where they are exposed to ascitic fluid, which may affect the behaviour and responses of the cancer cells. This behaviour and responses could differ from those of cells in cultures in the lab that are exposed to cell culture media supplemented with growth factors. In an attempt to better replicate the normal environment of ovarian cancer cells and to investigate the effects of canertinib and PHA665752 on the morphology and growth of OVCAR-5 clusters and SKOV-3 compact aggregates under these conditions, experiments were carried out in the presence of 50% (v/v) ascitic fluids from advanced ovarian cancer patients and growth factor activated cells. The rationale of using 50% (v/v) ascitic fluid was to mimic the *in vivo* microenvironment which is characterised by undiluted ascitic fluid [597]. This is to ensure that the cells are exposed to

high levels of cytokines and growth factors present in the ascitic fluid. However, other studies have used a much lower concentration of ascitic fluid (5-10%), which is not akin to physiological environment of advanced ovarian cancer [598, 599].

Growth factor treated cells showed notable morphological changes upon treatment with the inhibitors when compared to untreated controls. OVCAR-5 clusters appeared smaller with the single inhibitors and the inhibitor combination compared to untreated controls (**Figure 4.1 A**). There was also notable cell debris in the combination-treated cells.

SKOV-3 cellular aggregates (**Figure 4.1 C)** were large and compact with smooth edges in the growth factor treated controls, but cells treated with canertinib had an irregular rim on the aggregates and cell debris was notable. Cells treated with PHA665752 alone did not show these features in SKOV-3 compact aggregates, but with the combination of both inhibitors smaller aggregates and single cells were present. These responses were not seen in the presence of ascitic fluid with both OVCAR-5 (**Figure 4.1 B**) and SKOV-3 (**Figure 4.1 D**) cultures. In these experiments the morphology of all clusters and aggregates were similar to the controls. These results suggest that ascitic fluid may be affecting the efficacy of canertinib and PHA665752.

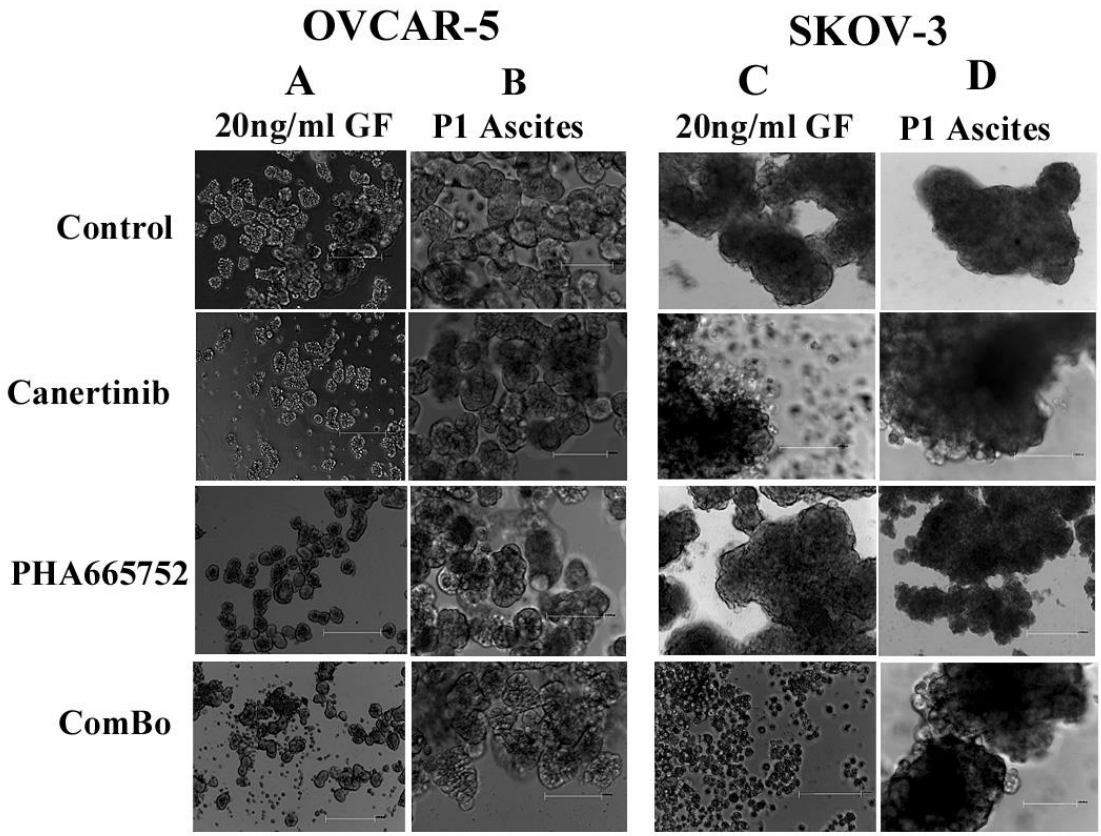


Figure 4.1. The effect of ascitic fluid on cell morphology of OVCAR-5 (A) and SKOV-3 (C) clusters and aggregates. There was a marked change in morphology when cells were treated with single or combination treatments of canertinib and PHA665752, in the presence of 20 ng/ml growth factors (GF) when compared to controls. In contrast OVCAR-5 (B) and SKOV-3 (D) cellular clusters exposed to ascitic fluid showed no obvious differences in morphology between the treatments and controls. Data are shown with ascitic fluid from patient 1. No obvious differences were also observed with ascitic fluid from patients 2 or patient 3 (data not shown). All experiments were independently performed at least three times with triplicates.

4.5.2 The effect of ascitic fluid on cell growth in the presence of TKIs.

To investigate the effect of ascitic fluid on the efficacy of the inhibitors OVCAR-5 and SKOV-3 clusters and aggregates were analysed after 48 hours of exposure to the inhibitors in the presence of ascitic fluid or GF. The expression of the proliferative cell nucleus antigen (PCNA) protein was also determined as a marker for cell proliferation in the S-phase of the cell cycle. As shown in **Figures 4.2** and **4.3** (and described earlier in the thesis), in the presence of the growth factors (GF) cell numbers of both OVCAR-5 clusters (**Figure 4.2**) and SKOV-3 aggregates (**Figure 4.3**), were significantly reduced in single inhibitor treatments but more markedly when the inhibitors were combined. The reduction of cell number correlated with a declined expression of PCNA.

In contrast, the cell numbers of OVCAR-5 cultures and SKOV-3 compact aggregates with the inclusion of ascitic fluid were higher than the GF treated cells, and the treatments with the single inhibitors or combination of inhibitors did not reduce the cell number. Furthermore, the expression of PCNA did not decrease. Similar results were obtained with the ascitic fluid from all 3 patients. These results suggest that the ascitic fluids may promote cell growth. They also demonstrate that in the presence of the ascitic fluid the inhibitors have no effect.

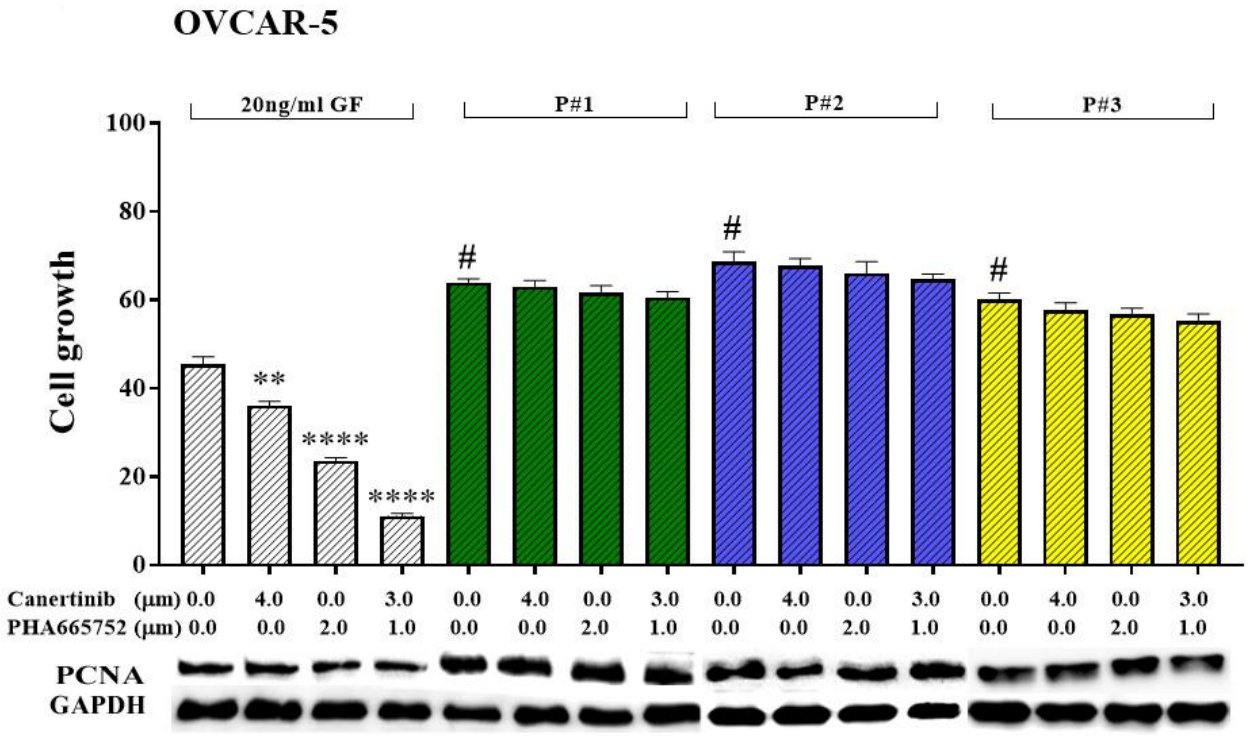


Figure 4.2. Ascitic fluids increased cell number relative to GFs and negated the effects of the inhibitors. The graphs indicate that canertinib and PHA665752 applied singly or in combination reduced the growth of OVCAR-5 cellular clusters, activated by 20 ng/ml growth factors (also as detailed earlier in the thesis). In contrast cell number increased in cultures that included 50% (v/v) ascitic fluid. Expression of PCNA was largely unaffected by the inhibitors in the presence of the ascitic fluid. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentration.

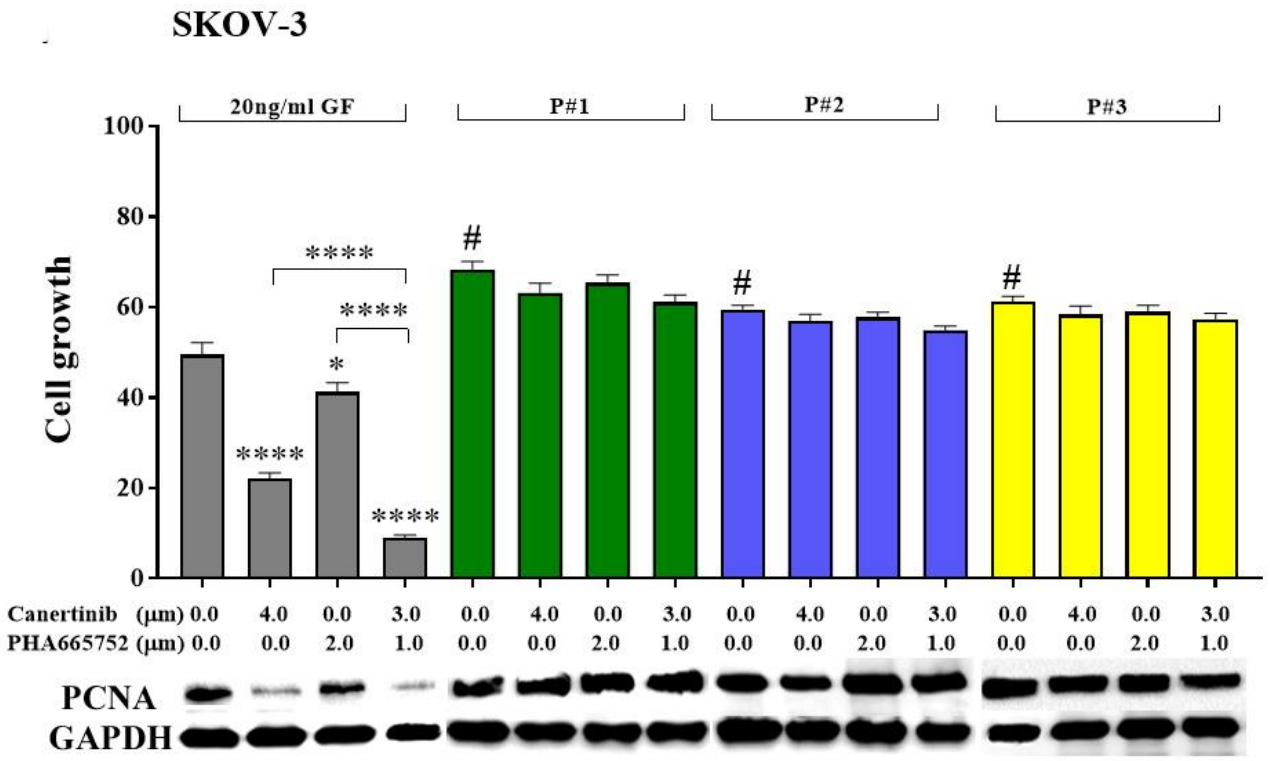


Figure 4.3. Ascitic fluids increased cell number relative to GFs and negated the effects of the inhibitors. The graphs indicate that canertinib and PHA665752 applied singly or in combination reduced the growth of SKOV-3 compact aggregates, activated by 20 ng/ml growth factors (also as detailed earlier in the thesis). In contrast cell number increased in cultures that included 50% (v/v) ascitic fluid. Expression of PCNA was largely unaffected by the inhibitors in the presence of the ascitic fluid. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

4.5.3 Ascitic fluid may compromise the Alamar blue dye assay

To investigate the effect of ascitic fluid on cellular metabolism in cell clusters and aggregates during treatment with the inhibitors, an Alamar blue dye assay was performed. As described earlier in the thesis Alamar blue is a non-toxic dye which is readily taken up and reduced in a redox reaction in viable cells. The reduced form of the Alamar blue dye has a pink colour which is then excreted into the cell culture media. This is a widely used technique to determine cell viability [600]. In the current experiments cells were exposed to the inhibitors either singly or in combination for 48 hours in the presence of 50% (v/v) ascitic fluid or GFs.

Cellular metabolism in both cell lines was very low in ascitic fluid treated cells, compared to GF treated cells, (**Figure 4.4 A** and **B**). This may suggest that the ascitic fluid may compromise cellular metabolism. There are two possible scenarios that could explain these results. First, the ascitic fluid may compromise the cellular activity and make the cells quiescent. However, this notion is not supported by the increase in cell number described in the previous section. The second scenario is that components in the ascitic fluid may bind to Alamar blue dye and that subsequently limits the movement of dye into cells.

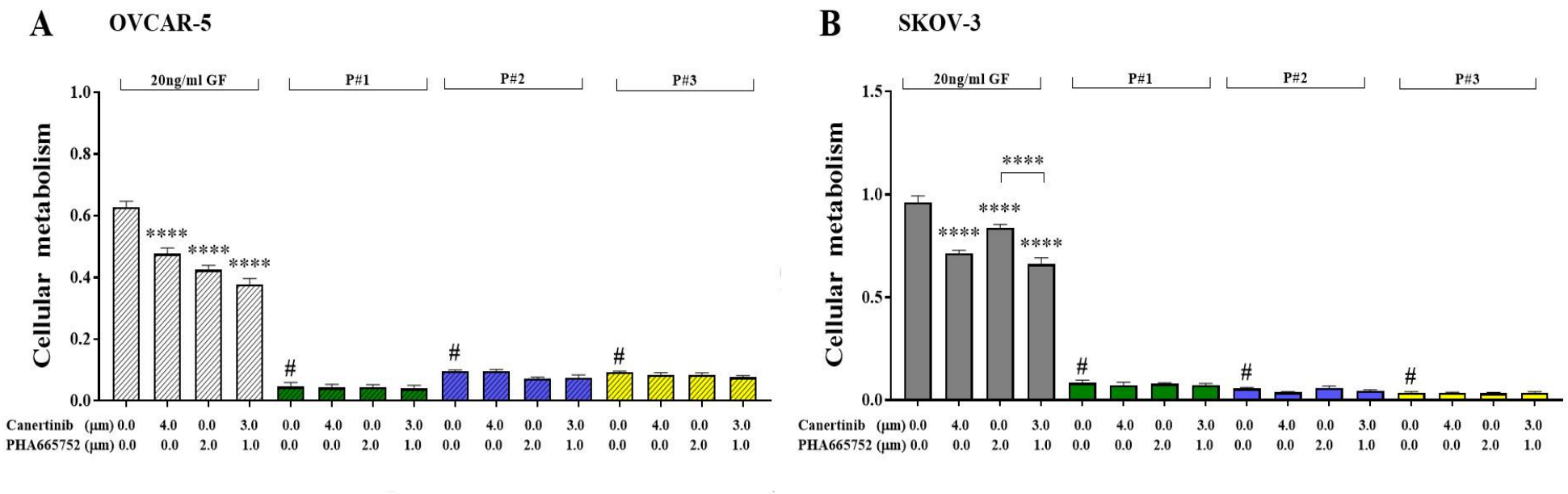


Figure 4.4. Ascitic fluids reduced cellular metabolism as indicated with an Alamar blue assay. Clusters of OVCAR-5 (A), and compact aggregates of SKOV-3 (B) cell lines showed a significant reduction in cellular metabolism, when treated with inhibitors singly or combination, in the presence of 20 ng/mL of GF, compared with untreated cells. The same cells show a significantly lower metabolism in the presence of ascitic fluid from each of the 3 patients. All experiments were independently performed at least three times with triplicates.). Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

4.5.4 Ascitic fluids compromised the uptake of PHA665752 in cell clusters and aggregates

Like most small molecule inhibitors, canertinib and PHA665752 are hydrophobic and are thought to move freely across the plasma membrane [601]. The inhibitors then bind to the ATP binding site of the tyrosine kinase domain located on the cytoplasmic side of the target receptor. As a result, the autophosphorylation of specific tyrosine residues is inhibited with subsequent inhibition of the associated signalling cascade. As described in Sections 4.5.2 and 4.5.3, the inhibitory effect of canertinib and PHA665752 was negated when ascitic fluids from 3 ovarian cancer patients were present. In order to determine why the ascitic fluids had this effect, the cellular uptake of PHA665752 was investigated to determine whether the ascitic fluid may prevent uptake of the inhibitors into the cells. PHA665752 shows fluorescent properties upon excitation with 488 nm light. This allows the determination of its uptake into cells using a flow cytometry.

After 48 hours of exposure to PHA665752 in the presence of ascitic fluids, the amount of PHA665752 in OVCAR-5 and SKOV-3 cells was significantly lower compared to that in cells that had been incubated in a serum free media (SFM) (**Figure 4.5. A** and **B**). This suggests that the ascitic fluids may affect the movement of the inhibitor into the cells.

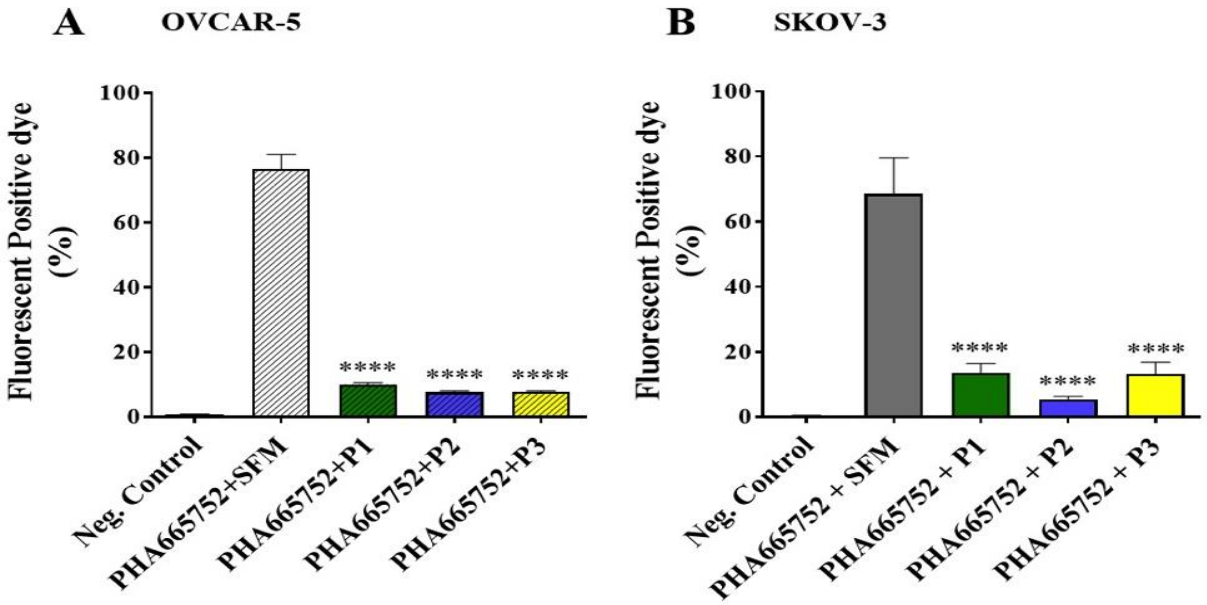


Figure 4.5. The cellular uptake of PHA665752 in the presence and absence of ascitic fluid. Clusters of OVCAR-5 (A) and aggregates of SKOV-3 cells (B) show reduced uptake of PHA665752 upon exposure to 50% (v/v) ascitic fluids from 3 different ovarian cancer patients (for detailed methodology see **Figure 2.12B**). Negative controls were clusters and aggregates in SFM with no PHA665752 or ascitic fluid treatment. All experiments were independently performed at least three times with triplicates.). Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

4.5.5 Removal of ascitic fluids restored the cellular uptake of PHA665752

To investigate whether the uptake of PHA665752 resumed after removal of the ascitic fluid cells were treated with 50% (v/v) ascitic fluid for 24 hours, the ascitic fluid was washed off and the cells were incubated with the inhibitor in SFM for a further 24 hours for a total exposure time of 48 hours (the method as shown in **Figure 2.12 C**). The uptake of PHA665752 increased in both OVCAR-5 clusters (**Figure 4.6 A**) and SKOV-3 aggregates (**Figure 4.6 B**), relative to uptake in the previous section, given that levels of PHA667252 uptake were similar to cells in the serum free medium (SFM) (**Figure 4.6 A** and **B**). This result suggests that certain components in ascitic fluid may compromise the cellular uptake of PHA665752 but that this effect can be reversed with the removal of the ascitic fluid.

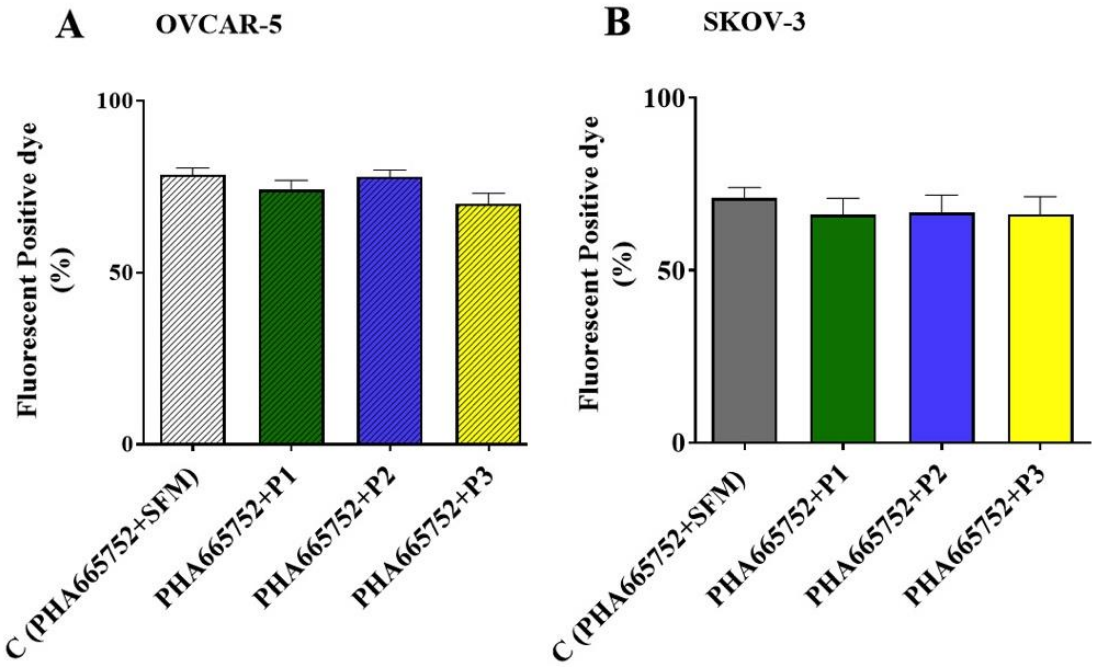


Figure 4.6. Removal of ascitic fluids restored the cellular uptake of PHA665752. OVCAR-5 clusters (A) and SKOV-3 aggregates (B) were able to take up PHA665752 when ascitic fluids were removed (For detailed methodology see **Figure 2.12 C**). All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

4.5.6 Treatment with PHA665752 for 24 hours before exposure to ascites reduced cellular uptake of the drug

To further examine the effect of the fluids on cellular uptake of PHA665752 the cells were incubated with the inhibitor prior to the inclusion of the ascitic fluids. Cells were exposed to PHA665752 for 24 hours before the introduction of ascitic fluid for a further 24 hours giving a total exposure time of 48 hours. The uptake of PHA665752 was markedly reduced after the ascitic fluids were introduced in OVCAR-5 clusters (**Figure 4.7 A**) and SKOV-3 compact aggregates (**Figure 4.7 B**). This may suggest that ascitic fluids may facilitate the efflux of the inhibitor out of cells.

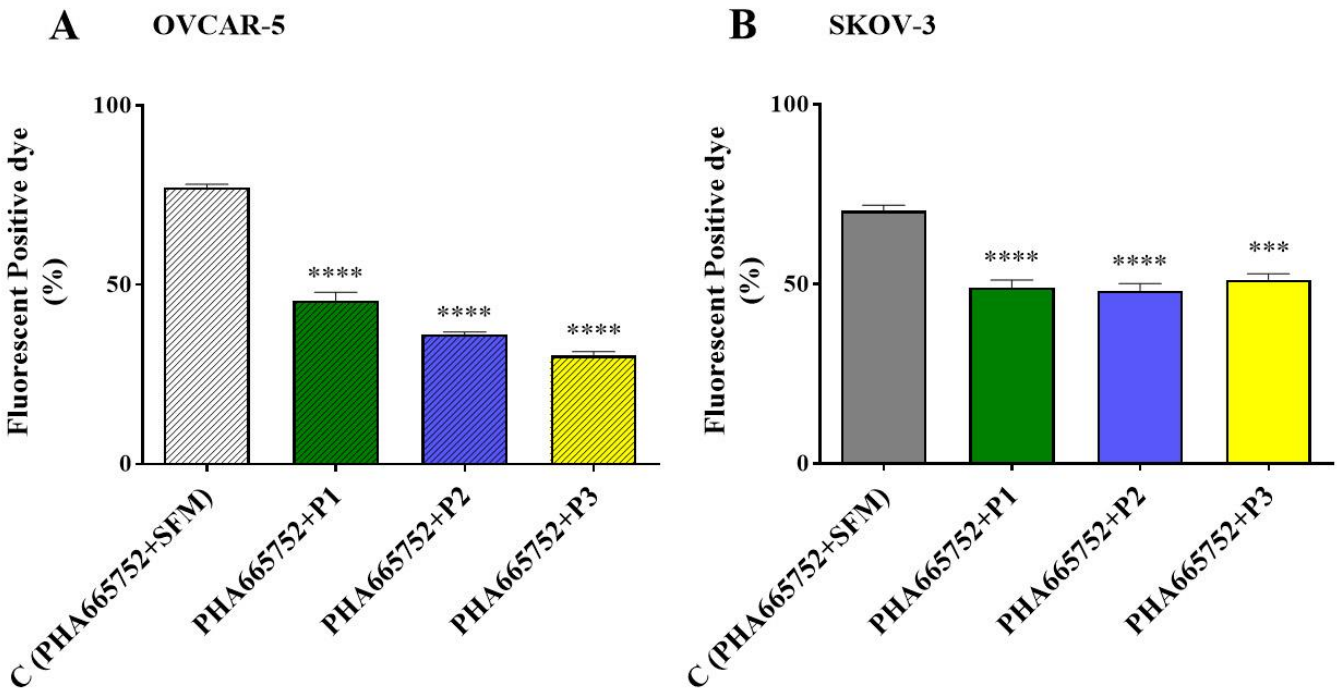


Figure 4.7. The cellular uptake of PHA665752 after an initial exposure of the cells to PHA665752 for 24 hours followed by exposure to ascitic fluids. OVCAR-5 clusters (A) and SKOV-3 aggregates (B) contained significantly less PHA665752 after they were exposed to ascitic fluid (methods shown in **Figure 2.12 D**). All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

4.5.7 Protein serum albumin compromise the cellular uptake of PHA665752

The above results suggest there is a possible interaction between PHA665752 with components in the ascitic fluid. These components could be possibly binding to the inhibitor and subsequently reducing the amount of PHA665752 that is taken up into the cell and/or affecting efflux from the cell. One candidate for such an interaction is human serum albumin protein. Levels of serum albumin are high in ascitic fluids obtained from patients with ovarian cancers [170]. Furthermore several anti-cancer drugs, including tyrosine kinase inhibitors, are known to bind to serum albumin [602] and as a result the efficacy of these inhibitors can be compromised.

In light of this an investigation of the effect of serum albumin on the uptake of PHA665725 was carried out. Bovine serum albumin (BSA) was used as a protein representative of human serum albumin as the two proteins share 78.1% of amino acid sequence identity [603] which may be sufficient for reliable binding properties. Moreover, BSA is frequently used as a model protein due to its structural homology with HSA [604] and a study by Bourassa and colleagues demonstrated that both proteins have similar binding properties even though the binding of Tamoxifen (an anti-tumour drug used for treatment of oestrogen receptor positive breast cancer) with BSA was less prominent than that of HSA [603]. The levels of BSA in FBS is approximately 35.6 mg/mL, which is close to the normal concentration of BSA in plasma (35-55 mg/mL) [605]. Plasma levels of HSA are within the range of 35-50 g/L. In light of this clusters and aggregates of OVCAR-5 and SKOV-3 ovarian cancer cell lines were exposed to PHA665752 in media supplemented with different concentrations of BSA (using the method described in **Figure 2.12 E**).

The uptake of PHA665752 in OVCAR-5 cellular clusters (**Figure 4.8 A**) and SKOV-3 compact aggregates (**Figure 4.8 B**) was found to be significantly reduced with increasing concentrations of BSA, relative to the control of serum free media. While I have assayed only PHA665752 it is possible that the non-specific binding capabilities of serum albumin may mean that it could play a role in reducing the cellular uptake of other tyrosine kinase inhibitors and thus that the presence of serum albumin may thus affect the efficacy of the drugs.

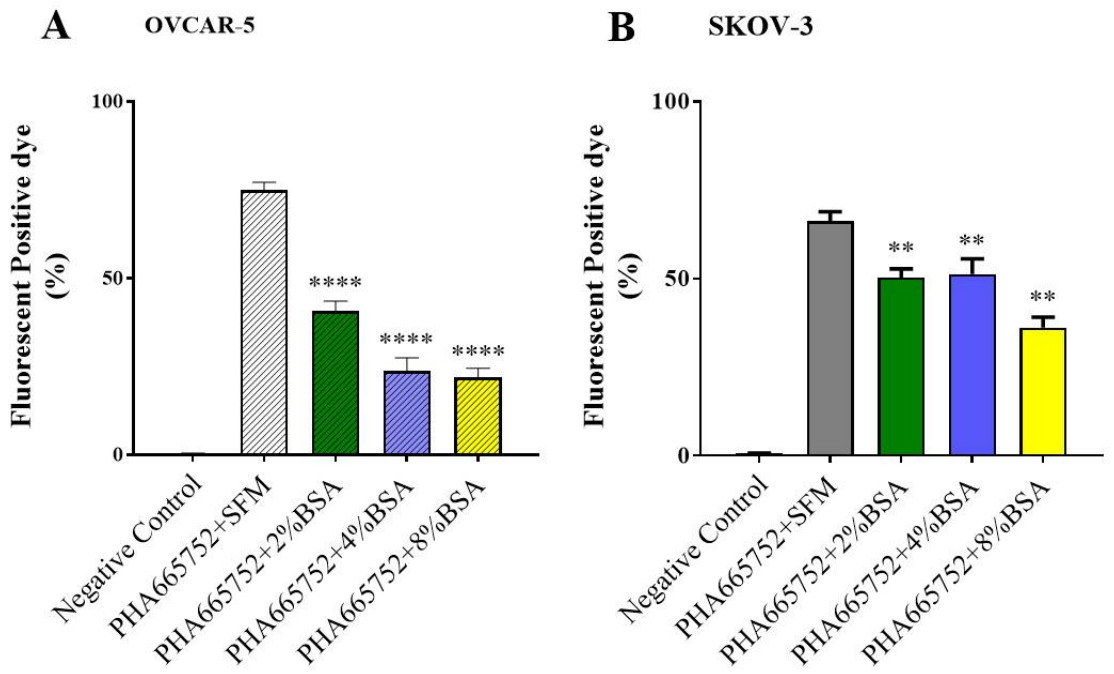


Figure 4.8. BSA reduced the uptake of PHA665752 into OVCAR-5 cellular clusters (A) and SKOV-3 compact aggregates (B), in a concentration dependant manner. This assay was carried out using the methods shown in **Figure 2.12 E**). Negative controls were cellular clusters and aggregates in serum free media SFM and no drug. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

4.5.8 Different concentration of PHA665752 affected the cellular uptake in the presence of 4% BSA

To investigate whether the inhibition of the uptake of PHA665752 by BSA could be overcome by increasing the concentration of the PHA665752, a dose response study was conducted in the presence of 4% BSA. This BSA concentration is a physiologically relevant one in the ascitic fluid of ovarian cancer patients [605]. For this assay the control cells were activated with media supplemented with a mixture of growth factors (GF). An increased concentration of PHA665752 led to elevated uptake of the inhibitor in both GF and BSA stimulated cells. However, in order to reach 80% of the cellular uptake of PHA665752 in OVCAR-5 cell clusters with 4% BSA 8 µM PHA665752 was required compared to 2 µM in GF supplemented media (**Figure 4.9 A**). Similar results were obtained with SKOV-3 cell aggregates (16 µM compared to 4 µM) (**Figure 4.9 B**). The increased uptake of PHA665752 was correlated with the reduction of growth activity.

In OVCAR-5 cell clusters, the concentrations of PHA665752 that reduced cell number by 50% (EC 50%) were 1.0 µM and 16 µM in GF and 4% BSA supplemented media respectively (**Figure 4.9 C**). In SKOV-3 cell aggregates, the EC 50% were approximately 2 µM and 32 µM with a GF medium and 4% BSA, respectively (**Figure 4.9 D**). Thus despite similar rates of uptake of PHA665753 there was a greater decrease of cell number in the OVKAR-5 clusters.

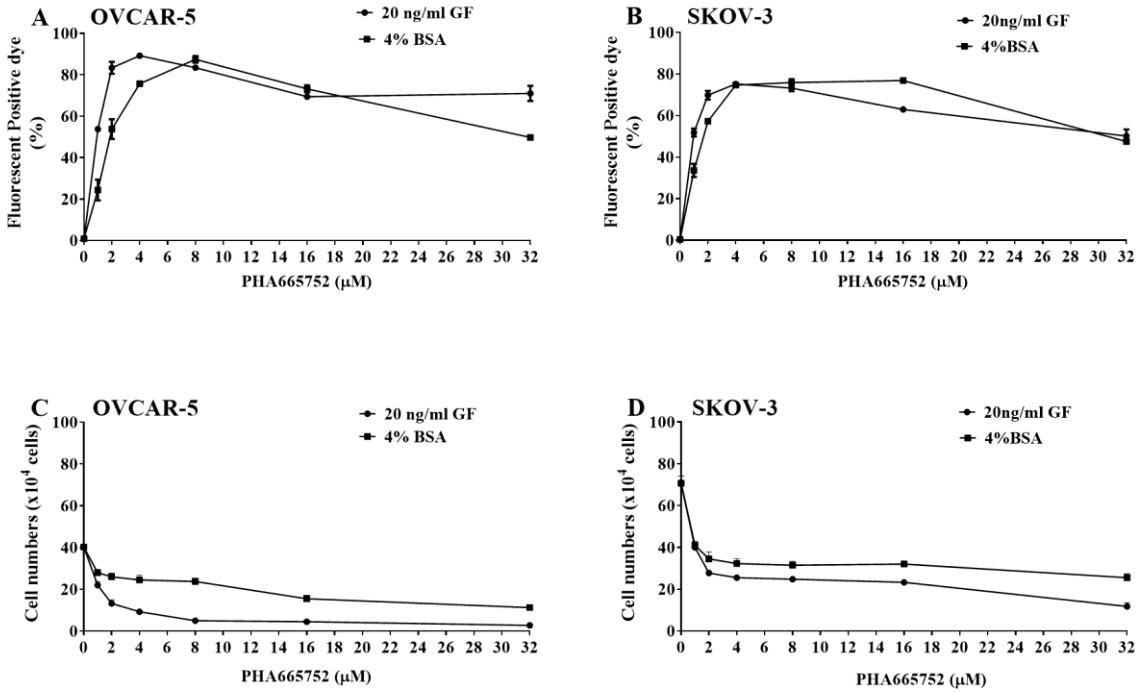


Figure 4.9. The effect of PHA665752 concentration on cellular uptake and cell number in the presence of growth factors (GF) and 4% BSA supplemented media. The cellular uptake of PHA665752 by ovarian cancer cell lines OVCAR-5 (A) and SKOV-3 (B), increased with increasing concentrations of PHA665752. Uptake reached a saturation plateau at about 8 μM. Cell numbers of OVCAR-5 (C) and SKOV-3 (D) cultures decreased in a concentration dependent manner in response to increasing concentrations of the inhibitor in the presence of 20ng/ml GF and 4% BSA (experimental methods were as shown in **Figure 2.12 F**). All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

4.5.9 The expression and phosphorylation of receptor tyrosine kinases were affected by ascitic fluids

Taken together, the above results suggest that there may be components in the ascitic fluid that bind to PHA665752 and thus prevent it from entering the cells and also enhance its efflux from the cells. It is unknown if a similar mechanism might also pertain to canertinib. However, drug resistance in ovarian cancer cells may also be attributed to the number of signalling proteins that are activated during exposure to ascitic fluids and subsequently cancer cells may thus be able to reduce the inhibitory effect of the drug(s). To investigate this possibility, the expression of EGFR, HER-2, and c-MET and their phosphorylation status were examined in the presence of ascitic fluid.

4.5.9.1 The effect of ascitic fluid on EGFR and p-EGFR

In both OVCAR-5 clusters (**Figure 4.10 A**) and SKOV-3 compact aggregates (**Figure 4.11 A**) there was a significant decrease of phosphorylated EGFR (p-EGFR) with single and combination treatments of canertinib and PAH665752 in both GF and ascitic fluid exposed cells. The ascitic fluid from all three ovarian cancer patients produced broadly similar results. The ascitic fluids from all three patients decreased the total expression of EGFR in SKOV-3 aggregates (**Figure 4.11 B**). However, the total expression of EGFR in the presence of ascitic fluid from patient 1 ascites was not significant in OVCAR-5 clusters. This was in contrast to the ascitic fluid from the other patients (**Figure 4.10 B).**

4.5.9.2 The effect of ascitic fluid on c-MET and p-MET

The combination of canertinib and PHA665752 significantly reduced the phosphorylation of MET protein (p-MET) in the GF exposed OVCAR-5 cell clusters (**Figure 4.12 A**). The combination also reduced p-MET in patient 1 ascitic fluid treated OVCAR-5 cell clusters. However, cells exposed to ascitic fluids from patients 2 and 3 showed less p-MET in all controls and treated cells (**Figure 4.12 A**). The inhibitory action of the two inhibitors showed a pronounced effect in p-MET in the GF and all ascitic fluid exposed SKOV-3 cells (**Figure 4.14 A**). The total expression of c-MET in ascitic fluids exposed OVCAR-5 (**Figure 4.12 B**) and SKOV-3 cells (**Figure 4.14 B**) were lower than the GF exposed cells.

4.5.9.3 The effect of ascitic fluid on HER-2 and p-HER-2

Canertinib applied alone and in combination with PHA665752 significantly reduced the phosphorylation of HER-2 (p-HER-2) in the GF and ascitic fluids of patients 1 and 3 treated SKOV-3 cell aggregates (**Figure 4.13 A**), but cells exposed to ascitic fluid from patient 2 had marginally reduced expression. The total expression of HER-2 was also reduced in canertinib treated cells both as a single treatment and with the combination. Again, the total expression of HER-2 in SKOV-3 cells treated with the ascitic fluid from the three patients was lower than the GF treated cells (**Figure 4.13 B**). OVCAR-5 cell clusters are a HER-2 negative cell line.

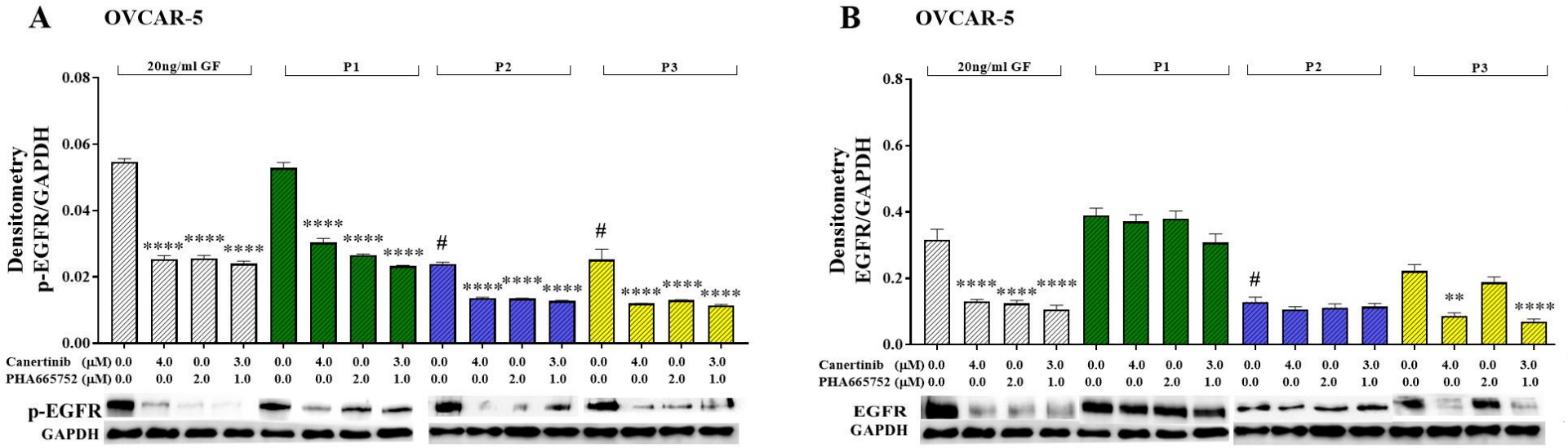


Figure 4.10. The effect of canertinib and PHA665752 on the expression and phosphorylation of EGFR in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of OVCAR-5 cellular clusters, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-EGFR, (B) EGFR, in the presence of ascites from advanced ovarian cancer patients. Each column colour represents ascites from a different advanced ovarian cancer patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

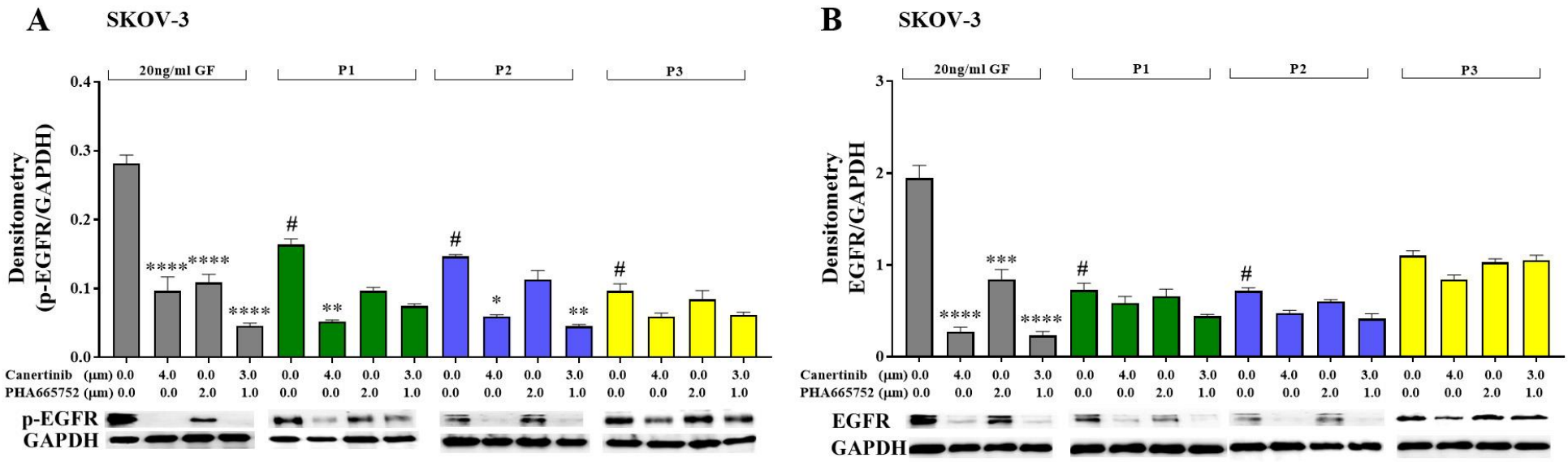


Figure 4.11. The effect of canertinib and PHA665752 on the expression and phosphorylation of EGFR in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of SKOV-3 compact aggregates, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-EGFR, (B) EGFR in the presence of ascites from advanced ovarian cancer patients. Each column colour represents ascites from a different advanced ovarian cancer patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

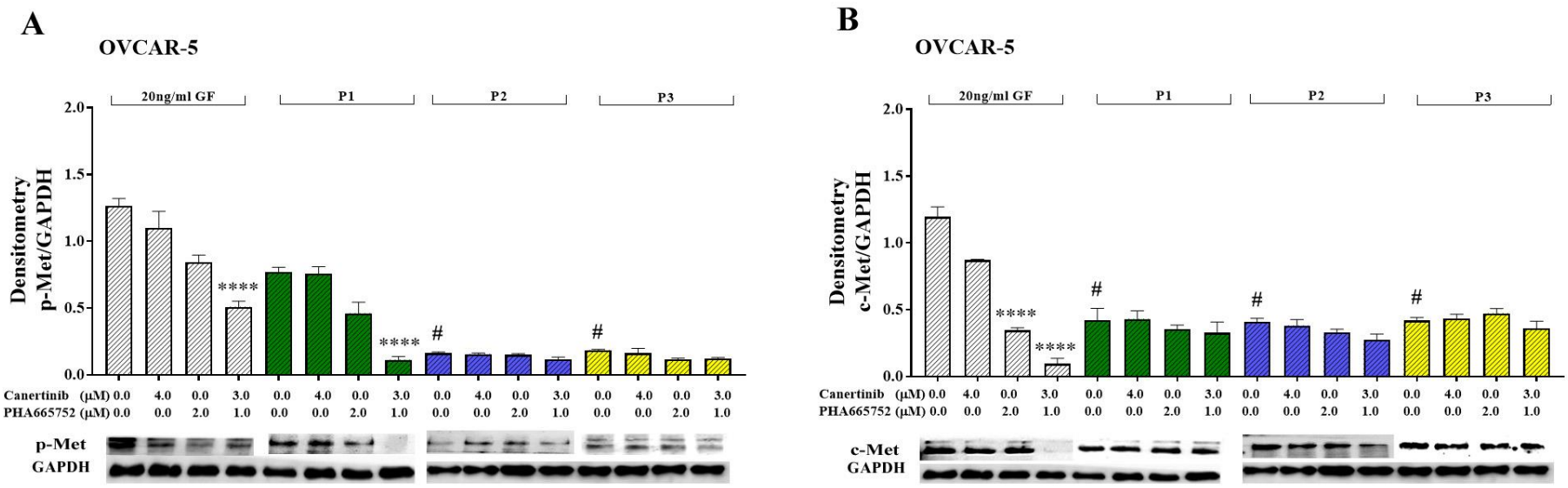


Figure 4.12. The effect of canertinib and PHA665752 on the expression and phosphorylation of c-MET in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of OVCAR-5 cellular clusters, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-MET, (B) c-MET, in the presence of ascites from advanced ovarian cancer patients. Each column colour represents ascites from a different advanced ovarian cancer patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

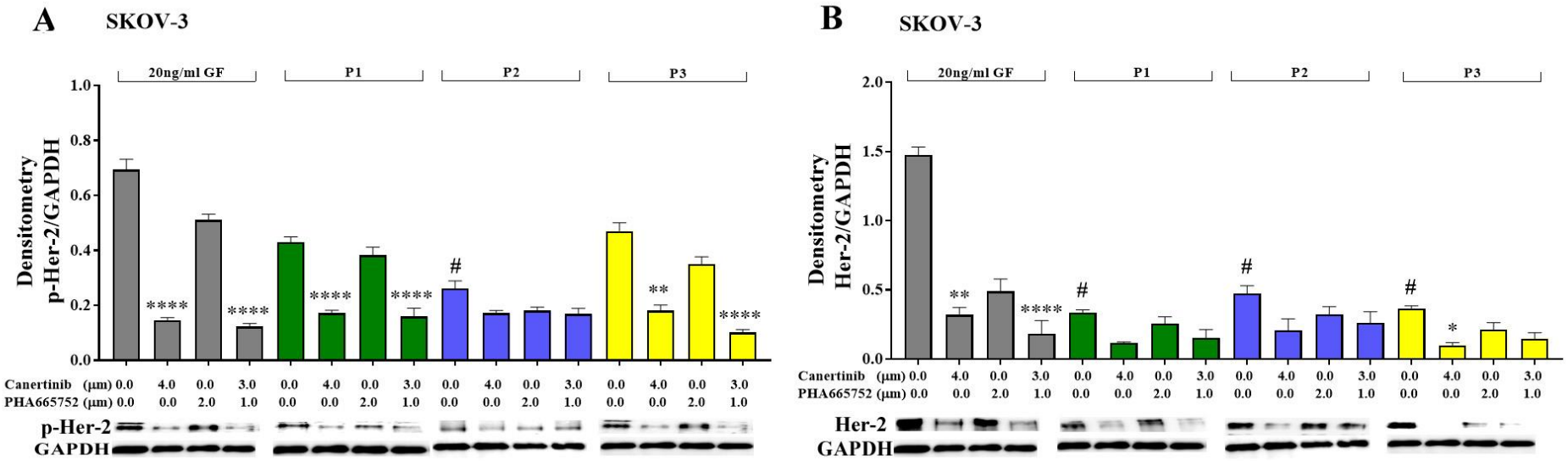


Figure 4.13. The effect of canertinib and PHA665752 on the expression and phosphorylation of HER-2 in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of SKOV-3 compact aggregates, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-HER-2, (B) HER-2 in the presence of ascites from advanced ovarian cancer patients. Each column colour represents ascites from a different advanced ovarian cancer patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

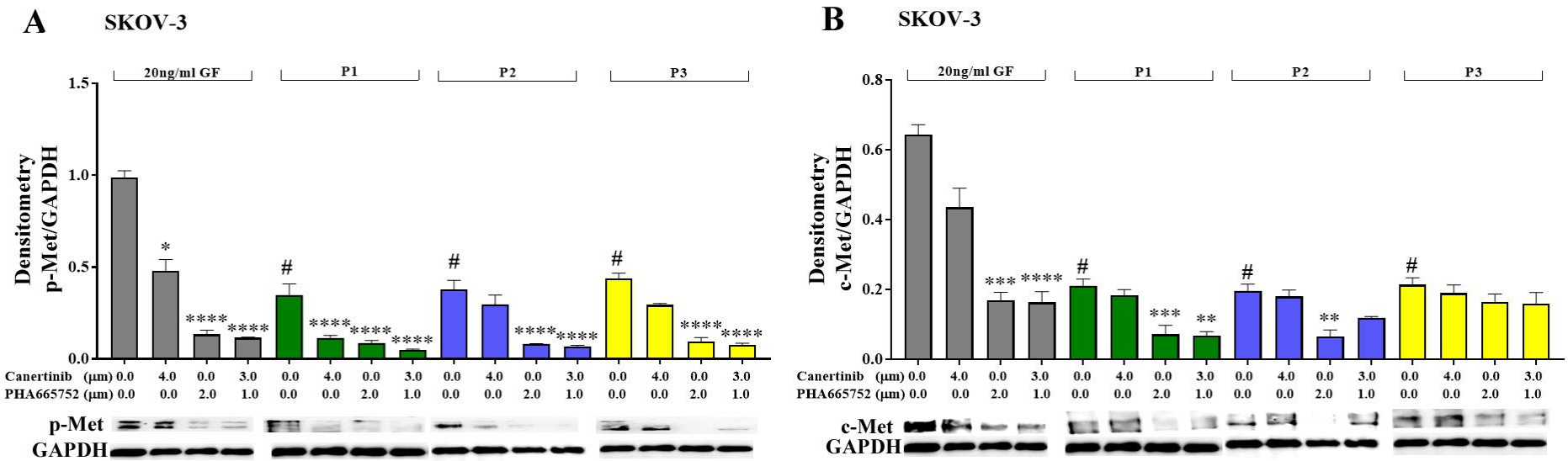


Figure 4.14. The effect of canertinib and PHA665752 on the expression and phosphorylation of c-MET in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of SKOV-3 compact aggregates, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-MET, (B) c-MET in the presence of ascites from advanced ovarian cancer patients. Each column colour represents ascites from a different advanced ovarian cancer patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

4.5.10 Ascitic fluids decrease the expression of downstream signalling proteins.

Akt and ERK are two of the downstream signalling proteins activated by EGFR, HER-2 and c-MET and these were investigated to further study the effects of inhibitors in the presence of ascitic fluid.

4.5.10.1 Akt and p-Akt

In comparison with the GF activated cells, the phosphorylated and total levels of Akt were significantly elevated in ascitic fluid activated OVCAR-5 cells (**Figure 4.15 A** and **B**). The levels of p-Akt and Akt in SKOV-3 cell aggregates were also significantly increased (**Figure 4.17 A** and **B**). Single and combined inhibitors did not reduce the Akt and p-Akt levels in the ascitic fluid activated OVCAR-5 and SKOV-3 cells. These responses were consistent with ascitic fluid from all three patients.

4.5.10.2 ERK and p-ERK

The phosphorylation and total expression of ERK was lower in both cell lines in cells that were in the ascitic fluid compared to the GF treated cells. Both p-ERK and ERK in both cell lines were significantly reduced in single and combination treatments of canertinib and PHA665752 with GF (**Figure 4.16 A** and **B** for OVCAR-5 and **Figure 4.18 A** and **B** for SKOV-3). However, single and combination of both inhibitors did not reduce the levels of p-ERK and ERK in OVCAR-5 cell clusters in the presence of ascitic fluid (**Figure 4.16 A and B**). The combination of both inhibitors reduced the levels of p-ERK in SKOV-3 cell aggregates in the ascitic fluid from patient 3 (**Figure 4.18 A** and **B**).

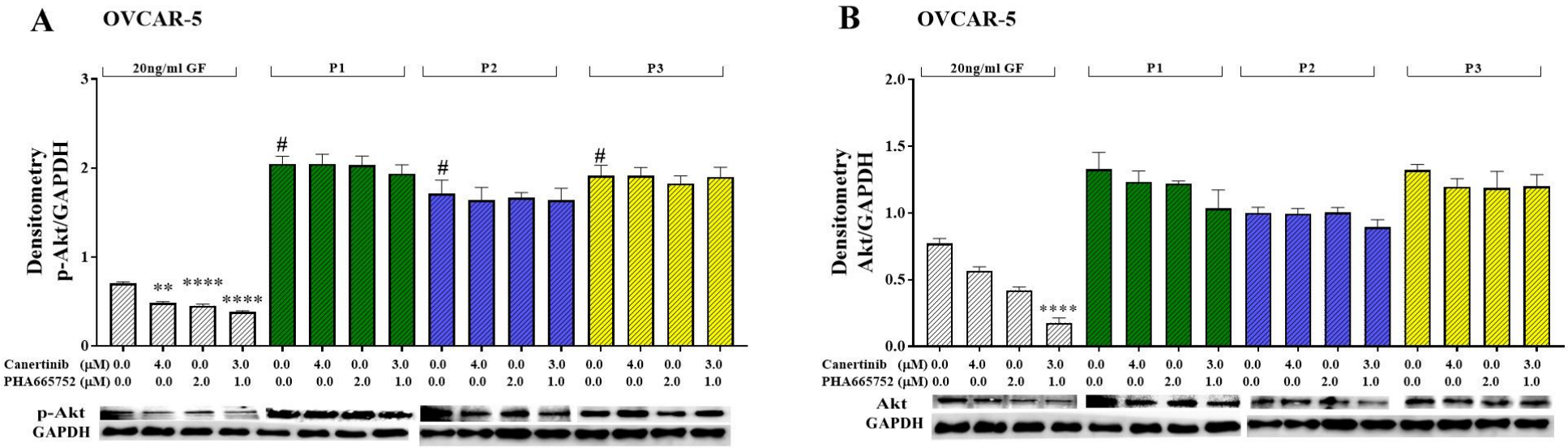


Figure 4.15. The effect of canertinib and PHA665752 on the expression and phosphorylation of Akt in the presence of ascitic fluids from three ovarian cancer patients. Western blots and densitometry analysis of OVCAR-5 cellular clusters show the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-Akt, (B) Akt in the presence of ascites from three ovarian cancer patients. Each column colour represents ascites from a different patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

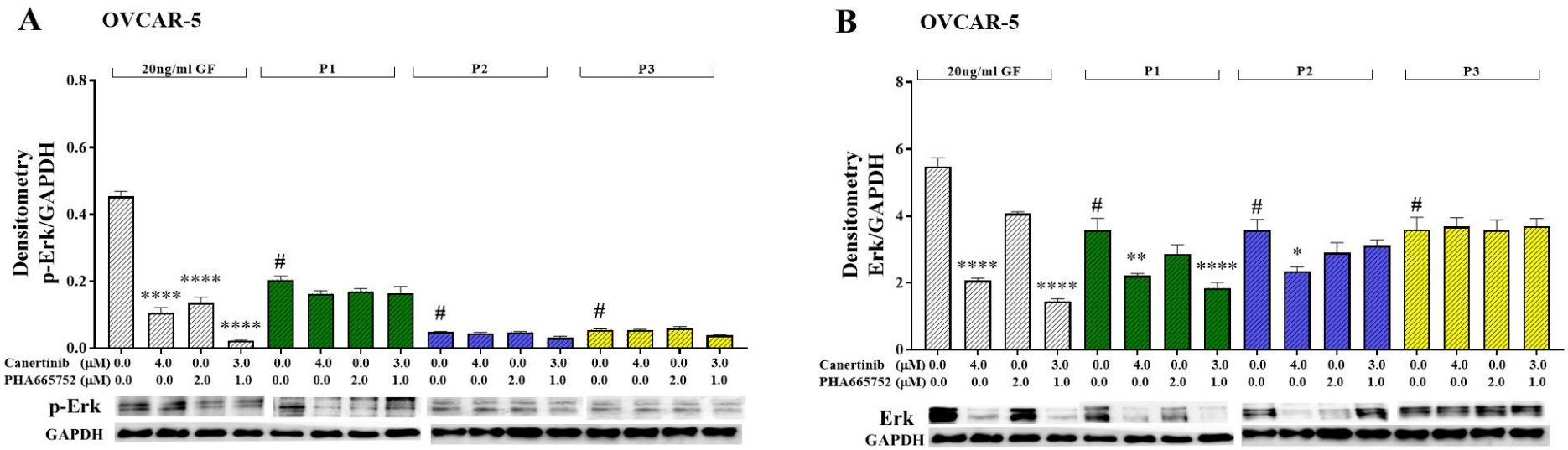


Figure 4.16. The effect of canertinib and PHA665752 on the expression and phosphorylation of ERK in the presence of ascitic fluids from three ovarian cancer patients. Western blots and densitometry analysis of OVCAR-5 cellular clusters show the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-ERK, (B) ERK, in the presence of ascites from three ovarian cancer patients. Each column colour represents ascites from a different patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

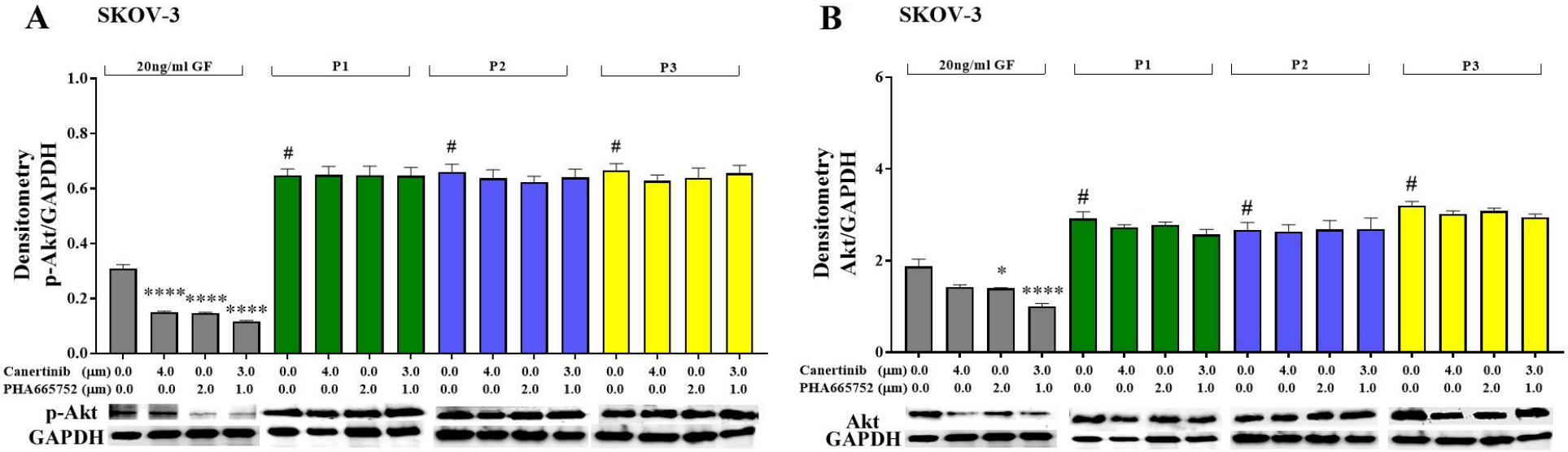


Figure 4.17. The effect of canertinib and PHA665752 on the expression and phosphorylation of Akt in the presence of ascitic fluids from three ovarian cancer patients. Western blots and densitometry analysis of downstream signalling molecules, Akt in SKOV-3 compact aggregates treated with canertinib and PHA665752 in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of SKOV-3 compact aggregates, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-Akt, (B) Akt in the presence of ascites from the patients. Each column colour represents ascites from a different patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control .

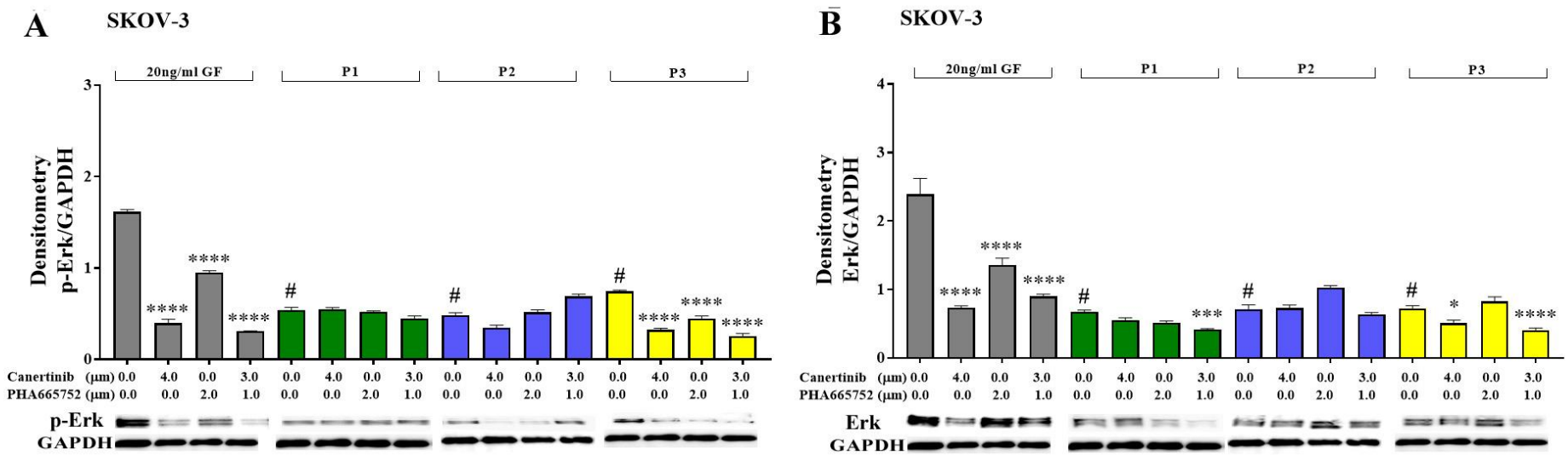


Figure 4.18. The effect of canertinib and PHA665752 on the expression and phosphorylation of ERK in the presence of ascitic fluids from three ovarian cancer patients. Western blots and densitometry analysis of downstream signalling molecules, ERK, in SKOV-3 compact aggregates treated with canertinib and PHA665752 in the presence of ascitic fluids from three ovarian cancer patients. Western blotting and densitometry analysis of SKOV-3 compact aggregates, showing the effects of canertinib and PHA665752, in single and combination, on total expression and phosphorylation of (A) p-ERK, (B) ERK, in the presence of ascites from the patients. Each column colour represents ascites from a different patient. All experiments were independently performed at least three times with triplicates. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

4.6 Discussion

In this chapter, the effects of ascitic fluids, obtained from advanced ovarian cancer patients, on the inhibitory action of two tyrosine kinase inhibitors in a 3D cell model of ovarian cancer cell lines were documented. The main findings were firstly that ascitic fluids promoted cell growth and sustained cell viability in the absence or presence of canertinib and PHA665752. Secondly that the expression of PCNA was maintained at a significantly high level in inhibitor treated cells in the presence of ascitic fluids. Thirdly that, ascitic fluids reduced the cellular uptake of PHA665752 and may also enhance efflux from the cell suggesting that a component in ascitic fluids could have an important role in influencing the bioavailability of this inhibitor, however, the effect on canertinib is still unknown. Fourthly that the ascitic fluids reduced the expressions of EGFR, HER-2, c-MET, ERK and p-ERK, but the levels of Akt and p-Akt were significantly elevated with or without inhibitors.

Ovarian cancer cells have been shown to increase their expression of tyrosine kinase receptors that are associated with the promotion of growth of tumours [464, 606]. Therefore, these receptors have been a major focus for the development of a target for drugs in the treatment of ovarian but also in several other types of cancers [84, 220, 313, 607-609]. It was established in the previous chapter, that the combination of canertinib with PHA665752 had a greater inhibitory effect on growth of 3D cell clusters than the single inhibitors alone [596]. In those experiments the ovarian cancer cells were exposed to growth factors supplemented in a culture medium, but this may not represent the true microenvironment that the cancer cells experience in advanced ovarian cancer patients.

Almost 37% of ovarian cancer patients present with ascitic fluids with malignant growth in the peritoneal cavity [165]. The biology underlining the ovarian cancer cells' behaviour when they are exposed to the ascitic fluid is under investigation. Ascitic fluid may provide a microenvironment that favours growth, survival and migration of the cancer cells [162, 168, 169]. Further the presence of ascitic fluid is associated with aggressive disease, drug resistance and a poor prognosis [572].

The mechanism(s) by which ascites contribute to aggressive disease, resistance to therapy and a poor prognosis is still poorly understood. There is evidence that cytokines play a crucial

role in the progression of ovarian cancer [169, 610]. There appear to be two main roles for cytokines in promoting the growth and progression of tumour cells. Firstly, they may directly induce growth, increase cellular adhesion and enhance angiogenesis acting as growth factors. Secondly, they may block the cell-mediated mechanisms that enable the recognition and destruction of tumour cells. Certain cytokines which are present in the normal ovarian environment have a significantly increased expression in the tumour microenvironment [610]. Lysophosphatidic acid (LPA) has been shown to be elevated in the blood and ascites of cancer patients and there is evidence of its role in the development and metastasis of ovarian cancer. It has been reported to be a key driver in the formation of inflammatory cytokines that contribute to the aggressive behaviour of ovarian cancer cells [583, 611, 612]. This may explain the growth promoting properties of the ascitic fluids which contain abundant growth promoting agents.

There have been limited studies investigating the effect of canertinib and PHA665752 under conditions that re-create as closely as possible those of the advanced ovarian cancer microenvironment. Ovarian tumours in the advanced stage proliferate on the surface lining of internal organs and abdominal walls. The tumours are constantly exposed to undiluted ascitic fluids, which are rich in a cocktail of proteins and bioactive small molecules [156, 168, 169, 180, 486, 498, 613]. Therefore, the response of ovarian cancer cells to these drugs in the presence of ascitic fluids is of interest.

This study, demonstrates that the morphology of OVCAR-5 cell clusters and SKOV-3 aggregates is notably changed upon exposure to the inhibitors when growth factors are present in a cell culture medium. This observation is consistent with previous studies on cell monolayers of ovarian cancer cell lines [219, 256, 257, 515]. In the present study, in the presence of ascitic fluids, cell clusters and aggregates are more compact than those treated with GF medium. This is consistent with the previous studies showing that ascitic fluids promote compact aggregate formation [7, 162, 614, 615]. Puiffe and colleagues [162] showed that ascitic fluids from different ovarian cancer patients had the same effect as FBS on the formation of compact spheroids of the ovarian cancer cell line OV-90 [162]. This may be explained by the presence of cytokines and growth factors in both FBS and ascitic fluid that may enhance cell-cell adhesion.

The morphology of the cell clusters and aggregates was not affected when the inhibitors were introduced in the presence of ascitic fluids. To the best of my knowledge, this is the first *in vitro* demonstration of the effects of tyrosine kinase inhibitors in the presence of ascitic fluids. There have been previous reports of ascitic fluids at lower concentrations stimulating ovarian cancer cells in *in vitro* conditions [162, 486]. In these studies the authors showed that, similar to FBS, a number of ascitic fluids increased growth in ovarian cancer cell lines, however, this characteristic was not universal and thus some ascitic fluids may not induce cell growth [162]. 5% FBS and 5% ascitic fluids were used in their experiments and the results were compared with cells in serum free medium [162]. Other studies have demonstrated that soluble factors in the ascites may be involved in the activation of survival pathways including Akt and ERK and that this may occur via integrins [598, 599]. Akt and ERK are important molecules in the process of cell growth, proliferation, and invasion. In context, the current study also showed that ascitic fluids can stimulate cells to produce high levels of PCNA. Furthermore in the presence of ascitic fluid the inhibitors canertinib and PHA665752 have no apparent effect on growth. These results are consistent with other studies on the effect of ascites on cell proliferation and growth [168, 616-618].

In an attempt to ascertain why the drugs have no inhibitory effect in the presence of the ascitic fluid, this study demonstrates that cellular uptake of the drug (PHA665752) was decreased and/or efflux of the drug was increased. Uptake of the drug returned after removal of ascitic fluid. It appears that there is some component in the ascitic fluid that affects the membrane permeability of the PHA665752. Ascitic fluids contain high levels of serum albumin due to the increased intraperitoneal permeability [619] caused by ovarian tumour growth [7, 619].

This study demonstrates that the presence of serum albumin protein in the ascitic fluids may have an impact on drug concentration in the cell which may explain the lack of effect of the inhibitors in the ascitic fluid. While this conclusion appears to contradict the findings in Chapter 3 where the drugs were still effective in the presence of 5% FBS it should be noted that the concentration of albumin in the 50% ascitic fluid would likely be higher than that in the 5% FBS that was used in the assays of Chapter 3.

Albumin has been shown to directly affect the pharmacokinetic and pharmacodynamic properties of anticancer drugs [620, 621]. The bioavailability of tyrosine kinases inhibitors, including gefitinib, erlotinib, lapatinib and afatinib have been shown to be affected by serum albumin in drug development studies [222, 479, 622-625]. Furthermore, the concentrations of the free unbound forms of these inhibitors in the plasma are below that which would be clinically active in ovarian cancer patients with EGFR and HER-2 positive tumours. Given that the structure of canertinib is similar to that of afatinib [609] it is possible that its bioavailability is similarly affected by albumin.

This study, also describes the changes of EGR, HER-2 and c-MET expression and phosphorylation after cells were exposed to ascitic fluid. The overall basal level of EGFR, HER-2 and c-MET in ascitic fluid treated cells was significantly decreased when compared to levels in GF exposed cells. After incubation with the inhibitors, the phosphorylation of these receptors was lower in the ascitic fluid exposed cells. This suggests that both canertinib and PHA665752 may affect the receptors but that their inhibitory action may be insufficient to elicit the growth arrest. One possibility is that additional signalling proteins could become activated and thus overrides any effect of the inhibitor [626].

This study demonstrates that levels of Akt and p-Akt are high in ascitic fluid exposed cells while the level of ERK and p-ERK does not change. This may suggest that the activation of Akt may be responsible for the growth promoting effect and acquired resistance to tyrosine kinase inhibitors. In comparison to benign plural fluids, tumour cells floating in the malignant ascitic fluid show over expression of Akt [627]. Akt can be activated and phosphorylated through several mechanisms such as the activation of cell surface receptors that utilise Akt in their downstream signalling processes. Lane and colleagues [599] showed that ascites from ovarian cancer patients promotes survival of ovarian cancer cells mainly via the activation of Akt [599]. Similar to results in this thesis, they show that inhibition of EGFR was not accompanied by inhibition of Akt and that ascites-induced activation of Akt was unaffected by the EGFR inhibitors PD153085 and AG1478, the EGFR inhibitors [599].

Growth factors that are abundant in ovarian cancer ascites contribute to the activation of Akt via the tyrosine kinase receptors such as EGFR and HER-2 and hence promoting cell survival

[628]. However, in this thesis, the tyrosine kinase inhibitors were able to partially inhibit the expression and phosphorylation of EGFR and HER-2 receptors but not Akt. This may suggest that an alternative mechanism for the activation of Akt may be utilised in ovarian cancer ascites.

Akt could also be activated through G-protein-coupled-receptors. These receptors have been implicated in tumour growth and cancer cell survival [583]. One ligand for these receptors is LPA which has been found to promote cell survival via signalling through PI3K/Akt pathway in ovarian cancer [629]. LPA has been shown to act as a ligand for four distinct receptors, LPA1, 2, 3, and 4 of which LPA2, 3 and 4 have been shown to be highly expressed in ovarian cancer ascites [630]. These receptors can couple with many G-proteins including G_q, G_i, and $G_{12/13}$. The coupling of LPA receptors with G_i can activate the PI3K/Akt pathway and the levels of LPA have been shown to be highly expressed in ovarian cancer ascites [631]. Activation of Akt may also be achieved through the interactions of ECM proteins with cell surface integrins leading to the activation of integrin/FAK signalling [599, 632]. Ovarian cancer ascites has been shown to induce an instant phosphorylation of focal adhesion kinase (FAK) which in turn is a key activator of Akt phosphorylation.

Matte, et al. [486], have demonstrated that OC ascites enhanced migration of patient derived human peritoneal mesothelial cells (HPMCs) by c-MET RTK phosphorylation via HGF stimulation, (which is abundant in most ascites), and EGFR activation, which in turn activates ERK1/2 and Akt intracellular signalling pathways [486]. These findings are consistent with results from this thesis showing the upregulation of Akt and p-Akt in OVCAR-5 and SKOV-3 clusters and compact aggregates. The inhibition of Akt activity is an area that warrants future investigation, for example a study of a combination of canertinib, PHA665752 and an Akt inhibitor for Akt in ovarian cancer cells exposed to ascitic fluids. The role of LPA in the activation of ovarian cancer cell growth also warrants further investigation to determine whether the presence of LPA activates Akt under the cell culture conditions used in the present study and whether inhibition of LPA in the ascites may enhance the cellular response to tyrosine kinase inhibitors or not. Given that ascitic fluids were from patients with high grade papillary serous ovarian carcinoma, and the fact that the response to the inhibitors was

similar in all three fluids, it would be of interest to compare these with ascitic fluids from patients with other subtypes.

In summary, the results suggest that there may be two main factors in ascitic fluids that affect the cellular response to TKIs; first, possible serum albumin binding to TKIs, which may prevent the drugs from freely entering the cells (and also enhancing possible drug efflux from the cells), and second an increased expression and activation of Akt could overcome the inhibitory effect of TKIs when ascitic fluids are present.

It is acknowledged that increasing the concentrations of TKIs used in this study may not be feasible in a clinical setting due to possible adverse side effects. However, the results presented in this chapter may highlight the need for a novel approach to drug delivery platform of the TKIs to overcome the effects of components in the ascitic fluid. One limitation of this study is the small number of ascitic fluids and ovarian cancer cell lines that were used due to resource and time constraints. Clearly further studies in this area are warranted.

CHAPTER FIVE

AN INVESTIGATION OF THE EFFECTS OF CANERTINIB AND PHA665752 ON CELL ADHESION OF THE TWO OVARIAN CANCER CELL LINES TO A COLLAGEN MATRIX.

5.1 Introduction

Ovarian cancer progression is a complicated multistep process in which cancer cells can exfoliate from their primary site of origin (the ovary) into the peritoneal cavity and move to and adhere to other organs. The process of adherence is important for progression of the disease and is a key factor with respect to the disease morbidity. Floating ovarian cancer cells in ascitic fluid can escape anoikis (a type of apoptosis induced by loss of attachment to the solid ECM substratum) [633, 634] through their adherence to the peritoneal surface of the abdominal wall, colon, liver, omentum, fallopian tubes, and bowel serosa [7, 367, 614, 635, 636]. The secondary growth of these cancer cells begins with their adherence to the surface of these organs. The cancer cells grow into larger tumour nodules, which can then compromise the normal function of the organs [636]. Patients with advanced ovarian cancer, for example, often present with bowel obstruction as a consequence of the tumour growing on the external wall of the gastrointestinal tract causing the blockage of bowel movement. This is a frequent cause of morbidity from ovarian cancer [637, 638]. As such, understanding the processes of adherence may provide important information about ovarian cancer progression. It is also possible that drugs that are able to delay the adhesion processes of the cancer cells may be of clinical significance.

The early stages of ovarian cancer cell adhesion occur as the cancer cells interact with the peritoneal lining of the abdominal cavity. The lining comprises mesothelial cells and extracellular matrix (ECM) proteins including collagen I, IV and laminin, blood vessels, lymphatics and nerve fibres [636, 639-641]. The mesothelial cells form a thin monolayer that covers the abdominal wall acting as a protective barrier against infections or tissue injury. Once cancer cells reach the abdominal wall, they adhere to the mesothelial lining and eventually disseminate deeper into organ tissue [571]. There are several possible stages that have been suggested for the implantation of ovarian cancer cells on the surface of peritoneal lining. Cancer cells may induce apoptosis in the thin layer of the mesothelial cells thereby removing the defensive barrier and exposing the basal lamina. This in turn leads to penetration of the basal lamina by the tumour cells [642]. The presence of soluble tumour-derived factors in the ascitic fluid can induce mesothelial retraction and disaggregation causing the basal lamina to be exposed, enabling the cancer cells to implant [643].

For the adhesion to be successful, the cancer cells must be in physical contact with either the mesothelial cells or the exposed collagens. As mentioned above, there is evidence to suggest that ovarian cancer cells can induce mesothelial cells to undergo apoptosis and that will expose the barrier between them and collagen matrix [642]. Integrins in the cancer cells can then facilitate contact with the ECM [644], binding to fibronectin, laminin and collagen [173]. The binding and activation of integrins can then trigger signal transduction pathways that play a role in the regulation of the cell cycle, cell shape, motility, and the addition of new receptors to the cell membrane [645].

Integrins have been suggested to play a role in co-activating numerous growth pathways during tumour angiogenesis [646]. It is thought that they can control the activity of receptor tyrosine kinases (RTKs) such as the vascular endothelial growth factor receptors (VEGFR) and the epidermal growth factor receptor (EGFR) [366, 647-649]. Crosstalk between integrin-mediated ECM signals and growth factors may contribute to control of the aggressive behaviour of cancer cells [650]. Collectively, the synergy between those signals may be a key driver of metastasis and tumour aggression [649, 651].

OVCAR-5 and SKOV-3 ovarian carcinoma cell lines express several adhesion molecules including integrins [652]. Both cell lines have been found to express high levels of β1 and β4 integrin subunits which can bind to several ECM components including laminin and collagen IV [653]. Integrins containing these subunits have been found to be responsible for the implantation of OVCAR-5 cellular clusters in the mesothelial lining of the abdominal cavity [364, 654] and the expression of these integrin subunits have been associated with a poor prognosis and aggressive disease [366, 367, 654]. As mentioned above, β4 integrin subunits have a specific affinity for laminin [655, 656]. In addition to their expression in OVCAR-5 and SKOV-3 ovarian cancer cell lines [657] an association with receptor tyrosine kinases EGFR, HER-2 and c-MET has been reported [372, 654, 658, 659]. These associations would be consistent with downstream cross-talk between RTKs and integrins and may underlie the co-localisation and clustering of the receptors upon activation and consequently the adhesion of the cells [370, 375, 376]. Integrin clustering recruits numerous downstream signalling proteins leading to the formation of focal adhesions. Cell-matrix adhesions differ in 2 and 3 dimension culture conditions [660]. The stable attachment of normal epithelial cells is to the basement membrane is maintained through the formation of hemidesmosomes as a result of

α6β4 integrin activation [661]. During wound healing and malignant development this integrin is phosphorylated causing the disassembly of hemidesmosomes. This in turn leads to a redistribution of α6β4 integrin to interact with the actin cytoskeleton where it contributes to cell migration and invasion via co-operating with tyrosine kinase receptors [375, 658].

In addition to their role in mediating cell adhesion, the overexpression of β4 integrin subunits in ovarian cancer cells can promote cell migration, proliferation and metastasis by controlling the localisation and action of ECM proteins [366]. Importantly some drug resistant cancer cells may occur due to the interaction between integrins and the ECM, and in fact it has been suggested that there may be cooperation between different integrins in the induction of drug resistance [645]. Some studies have shown that β4 integrins are involved in tumour propagation and drug resistance in NSCLC [645, 662], however, the resistance of matrix adherent ovarian cancer cells to treatments is thought to be due to a pro-survival response due to the interaction of β1and β4 integrin subunits with integrin linked kinase (ILK), and focal adhesion kinase (FAK) [645].

The α6β4 integrin has been shown to induce EGFR clustering in breast cancer cells [658] leading to selective activation of EGFR signalling thereby affecting chemotaxis or motility [658]. This clustering has been shown to be inhibited when a PI3K inhibitor was introduced, suggestive of a role of PI3K in inducing the clustering mechanism [663]. In addition, c-MET and its ligand HGF have been shown to increase peritoneal adhesion of OVCAR-3 and SKOV-3 ovarian cancer cell lines in an *ex vivo* cell culture model [528]. Inhibition of c-MET with the small molecule inhibitor INC280 (an orally bio-available c-MET inhibitor) reduced cell adhesion of these cell lines [528]. Other agents including PF-2341066 (c-MET-specific), Foretinib (c-MET and VEGFR-2), MK8033 (c-MET specific), DCC-2701 (c-MET/Tie-2/VEGFR-2), and SU11274 (c-MET specific) have been tested in pre-clinical trials of ovarian cancer and shown to reduce cell invasion, adhesion, metastasis and tumour burden in animal models [335, 345, 525, 664, 665]. These all implicate a complex interplay between integrins, receptor tyrosine kinases and cell adhesion and thus a role for these membrane proteins in metastasis.

In view of the above and the results described in earlier chapters an investigation was made of the effect of the two tyrosine kinase inhibitors on cell adhesion in the two ovarian cancer cell lines.

5.2 Hypotheses of this chapter

Canertinib and PHA665752 will inhibit integrin-mediated cell adhesion of ovarian cancer cell clusters and compact aggregates.

The effect on adhesion will occur due to the inhibition of the phosphorylation of EGFR/HER-2 and c-MET.

EGFR and HER-2 receptor tyrosine kinase will co-localise intracellularly with β4 integrin subunits, and the inhibition of the receptors will reduce the co-localisation leading to loss of adhesion.

5.3 Objectives of this chapter

1. To investigate the effect of EGF and HGF on the adhesion of ovarian cancer cell clusters and compact aggregates to collagen gel matrix.

2. To investigate the effect of canertinib and PHA665752 tyrosine kinase inhibitors on the adhesion of ovarian cancer cellular clusters and compact aggregates to collagen gel matrix in the presence of growth factors.

3. To investigate the downstream effects and internal co-localisation of EGFR/ HER-2 with β4 Integrin subunits.

5.4 Materials and methods

Materials and methods were as described in chapter 2 section 2.13.

5.5 Results

5.5.1 Expressions of β1 and β4 integrin subunits in OVCAR-5 and SKOV-3 cells

Initially the presence of β1 and β4 integrin subunits in OVCAR-5 cellular clusters and SKOV-3 compact aggregates was investigated by immunofluorescent imaging. Both types of cell lines exhibited high expression of β1 and β4 integrin subunits (**Figure 5.1 A, B, C and D**).

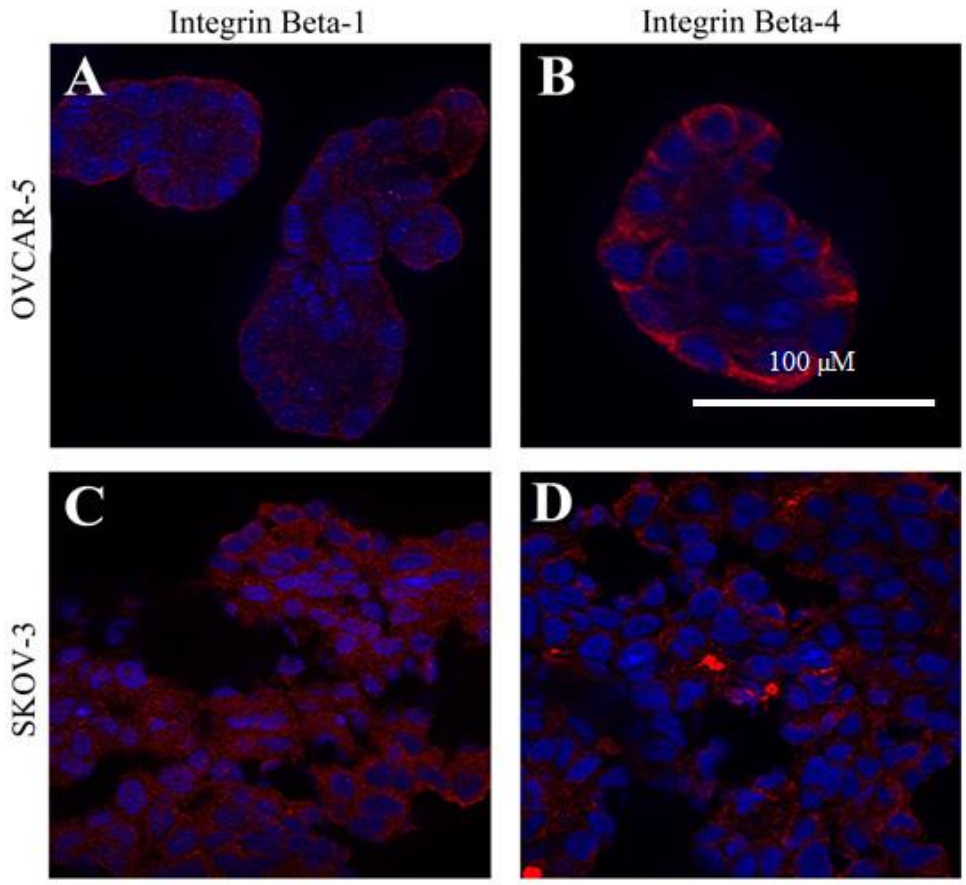


Figure 5.1.Immunofluorescent images of β1 and β4 integrin subunits in ovarian cancer cell lines. (A and B) OVCAR-5 cellular clusters and (C and D) SKOV-3 compact aggregates. DAPI stained nuclei are blue and the plasma membrane associated β1 or β4 integrin subunits are stained red.

5.5.2 The presence of growth factors enhances cell adhesion to a collagen gel matrix in the two ovarian cancer cell lines

As established in chapter 3, the presence of growth factors activates the EGFR, HER-2 and c-MET receptors, leading to increased proliferation and cell growth of OVCAR- 5 cellular clusters and SKOV-3 compact aggregates. Further, results presented in chapter 4 reported that ascitic fluid increased the growth of ovarian cancer cell clusters/aggregates. In the body ascitic fluid containing cancer cells is an environment that is rich in growth-promoting factors particularly EGF and HGF [46]. Hence the role of these growth factors in the adhesion of OVCAR-5 cellular clusters and SKOV-3 compact aggregates was investigated.

A dose-dependent increase in the adhesion of OVCAR-5 cellular clusters and SKOV-3 compact aggregates was observed with both EGF and HGF. This was more marked in the SKOV-3 cells than the OVCAR-5 cells (**Figure 5.2 A, C** and **B, D**). There were no significant differences between the 0.2 and 20 ng/mL of GF in OVCAR-5 clusters. However, SKOV-3 compact aggregates exhibited significant differences between the two different concentrations of EGF.

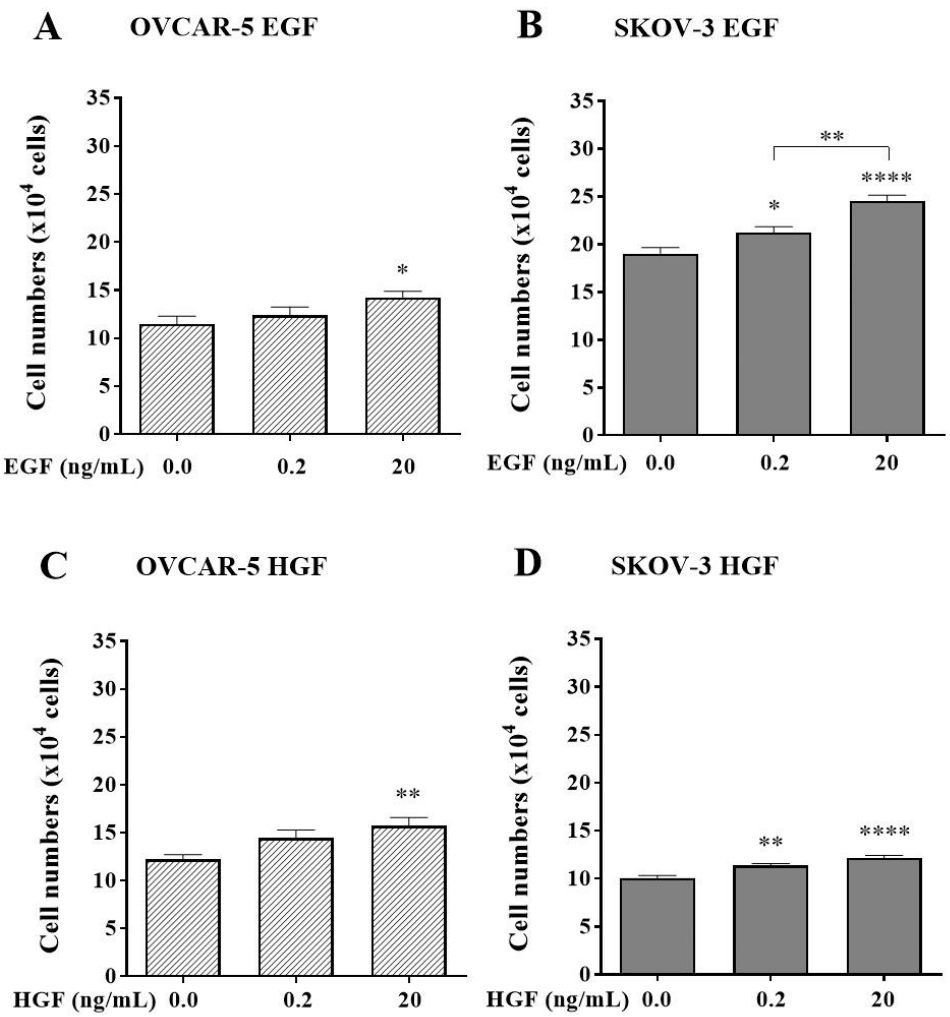


Figure 5.2. Effects of EGF and HGF on the adhesion of the OVCAR-5 and SKOV-3 ovarian cancer cell lines. Numbers represent the number of adherent OVCAR-5 (A, C) and SKOV-3 (B, D) clusters/compact aggregates treated with 0.2 and 20 ng/mL of EGF or HGF. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.3 Canertinib inhibits adhesion of OVCAR-5 clusters and SKOV-3 compact aggregates in a dose dependent manner

To investigate the effect of the tyrosine kinase inhibitor canertinib on adhesion, cells were exposed to different concentrations of the inhibitor for four hours (the average time for ovarian cancer cells to achieve maximum adherence [364]), in the presence of 0.2 or 20 ng/mL of the EGF - HGF combination (termed GF as described earlier). There was a concentration dependent decrease in the number of adherent cells of OVCAR-5 clusters (**Figure 5.3 A, C**) and SKOV-3 compact aggregates (**Figure 5.3 B, D**) in the presence of 0.2 or 20 ng/mL of GF with increasing concentrations of the inhibitor. This result was found irrespective of the concentration of GF. The question of whether this loss of adhesion is due to cell death or to other intracellular responses to the inhibitor will be addressed below.

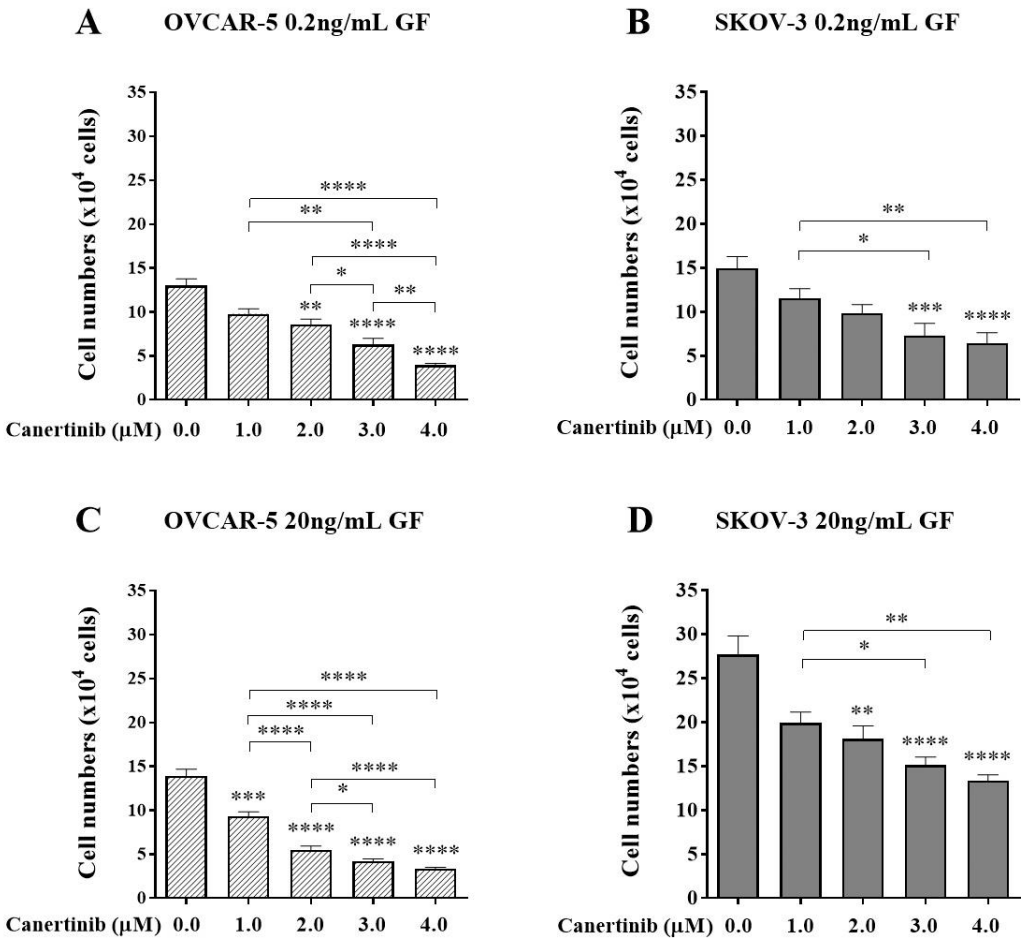


Figure 5.3. The effect of canertinib on cell adhesion of the two ovarian cancer cell lines OVCAR-5 (A, C) and SKOV-3 (B, D). Cell clusters and compact aggregates were treated with canertinib at concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 μM in the presence of 0.2 or 20ng/mL GF for 4 hours before a further 4 hours adhesion assay. Adherent cells were counted. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.4 PHA665752 inhibits adhesion of OVCAR-5 clusters and SKOV-3 compact aggregates in a dose dependent manner

To investigate the effect of the c-MET inhibitor PHA665752 on cell adhesion, cells were exposed to different concentrations of the inhibitor, in the presence of 0.2 or 20 ng/mL EGF /HGF combination (GF), for 4 hours before the adhesion assay was carried out and adherent cells were counted. There was a significant dose dependant decrease in the adhesion of OVCAR-5 cellular clusters (**Figure 5.4.A, C**) and SKOV-3 compact aggregates (**Figure 5.4.B, D**) irrespective of the concentration of growth factors in response to the presence of the inhibitor. This effect was more pronounced in OVCAR-5 clusters.

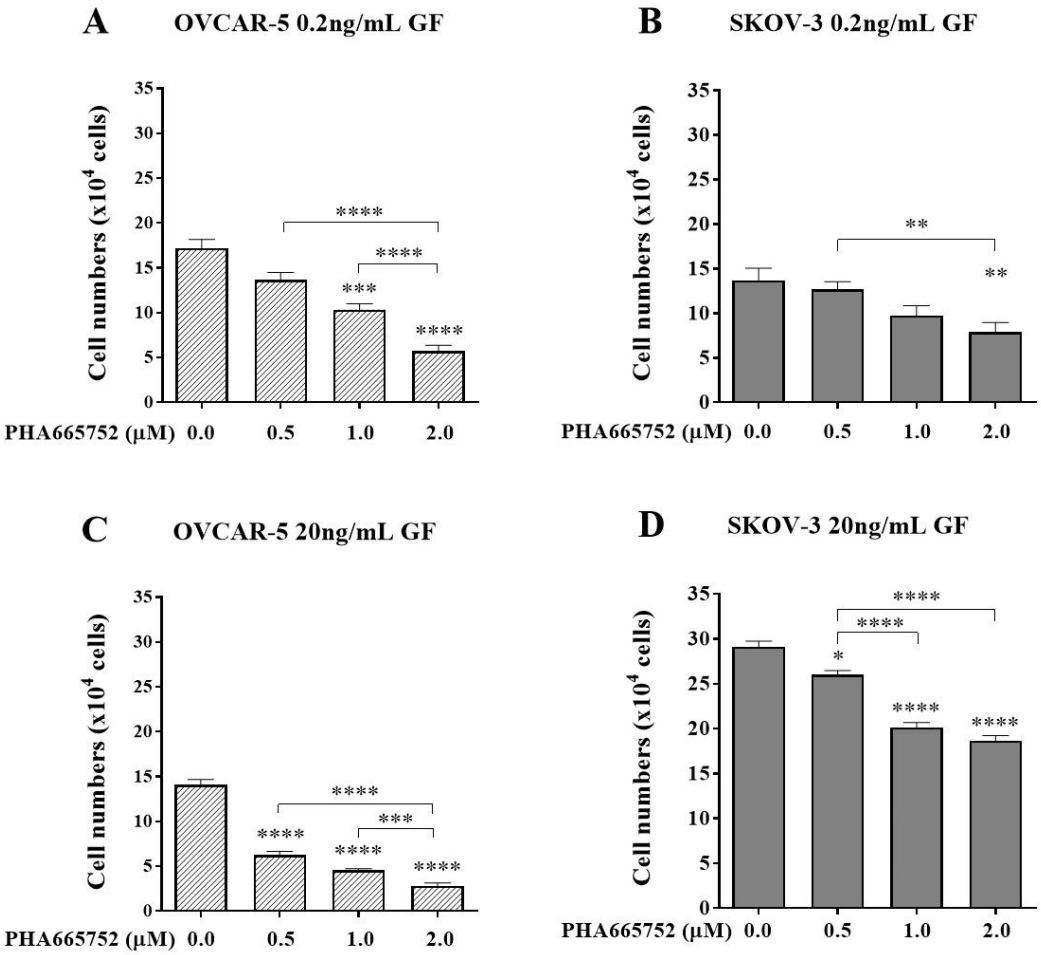


Figure 5.4. The effect of PHA665752 on cell adhesion of the two ovarian cancer cell lines. Number of adherent OVCAR-5 (A, C) and SKOV-3 (B, D) clusters/compact aggregates treated with 0, 0.5, 1.0, and 2.0 μM of PHA665752 in the presence of 0.2 or 20ng/mL GF. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

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5.5.5 A combination of canertinib and PHA665752 inhibits adhesion more effectively than single inhibitor treatments

Since the focus of this thesis is the effect of combining the two inhibitors, canertinib and PHA665752 on the two ovarian cancer cell lines, the effect of a combination of both inhibitors in the presence of 0.2 or 20 ng/mL combined growth factors (GF), on adhesion was investigated. As described above and here there was a significant decrease in cell adhesion with single inhibitors for both OVCAR-5 clusters (**Figure 5.5 A, C**) and SKOV-3 compact aggregates (**Figure 5.5 B, D**). A further reduction in cell adhesion was observed when cells were exposed to the inhibitor combination. There was no difference in the response with the different concentrations of growth factors.

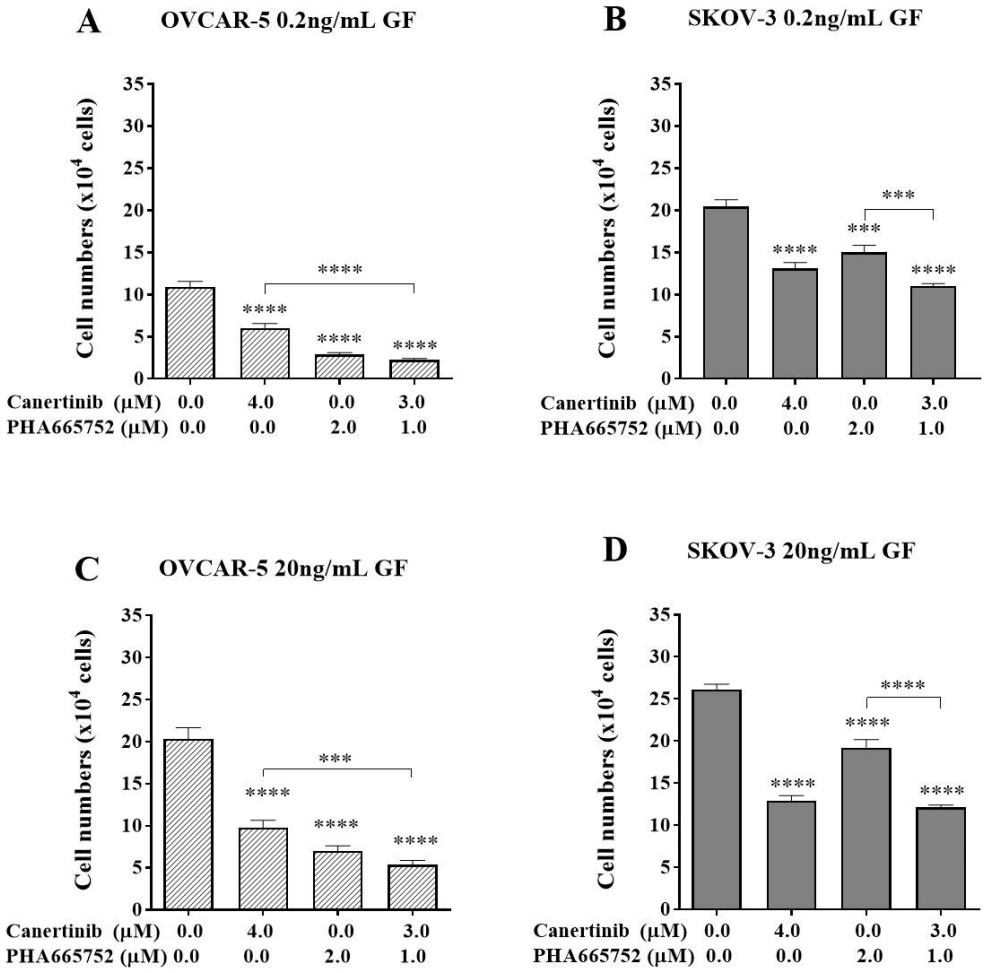

Figure 5.5. The effect of a combination of the two tyrosine kinase inhibitors, canertinib and PHA665752, on the adherence of OVCAR-5 clusters and SKOV-3 compact aggregates. Cell clusters and compact aggregates were treated with single and combination of canertinib and PHA665752, in the presence of 0.2 or 20ng/mL GF for 4 hours before a further 4 hours of the adhesion assay. Adherent cells were counted. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.6 Canertinib, alone or in combination with PHA665752 did not compromise cell growth and metabolism within 4 hours of cell adhesion assay

In order to confirm that the effect of the inhibitors, in single or combination was not due to them reducing cell numbers (in the 4 hour exposure prior to an adhesion assay) rather than affecting adhesion, an investigation of the effect of canertinib alone and in combination with PHA665752 on cell number and cellular metabolism was carried out. With both growth factor concentrations, 0.2 or 20 ng/mL, there was in the majority of cases no significant decline in cell number of OVCAR-5 clusters (**Figure 5.6 A, B**) or SKOV-3 compact aggregates (**Figure 5.6 E, F**) with the increased canertinib concentrations. However, SKOV-3 cells showed a slight, but significant, reduction in cell number with the combination of canertinib and PHA665752 (**Figure 5.6 E**). No significant effect on cellular metabolism was observed in both OVCAR-5 clusters (**Figure 5.6 C, D**) and SKOV-3 compact aggregates (**Figure 5.6 G, H**) in both 0.2 and 20 ng/mL GF. This suggests that canertinib is not compromising cell growth within the 4 hours timeframe but rather is inhibiting adhesion.

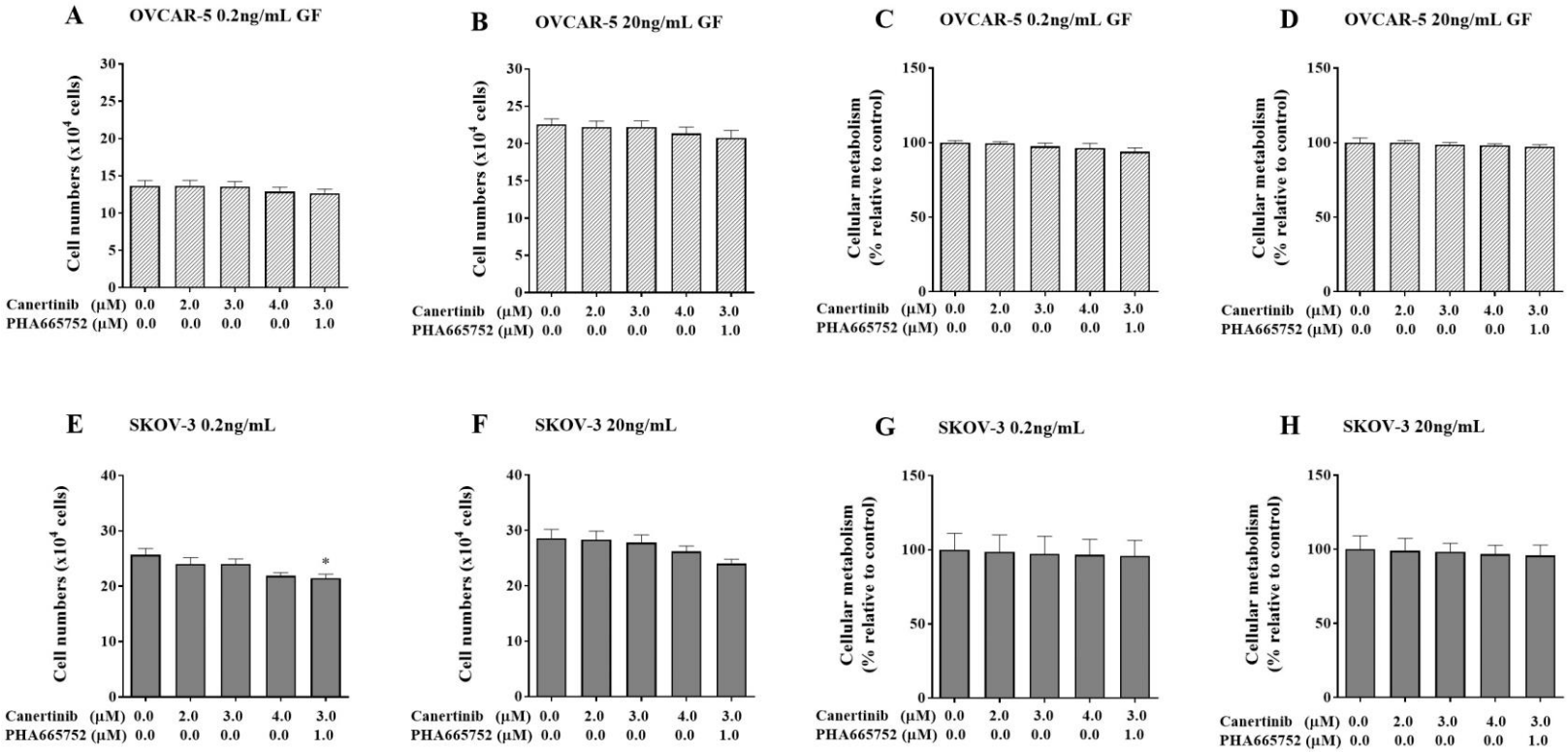

Figure 5.6. The effect of 4 hours incubation with different concentrations of canertinib alone or in combination with PHA665752 on the growth and metabolism of OVCAR-5 cellular clusters and SKOV-3 compact aggregates. Cell growth of OVCAR-5 (A and B) and SKOV-3 (E and F) exposed to single canertinib or in combination with PHA665752 in the presence of 0.2 or 20 ng/nL of GF. Metabolism of OVCAR-5 (C and D) and SKOV-3 (G and H) for the same conditions.Data are expressed as means ± S.E.M (n=9). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.7 PHA665752, alone or in combination with canertinib, did not affect cell growth or metabolism within the 4 hours of the adhesion incubation period

In order to investigate whether PHA665752 alone, or in combination with canertinib, affected cell number within the 4 hours' time frame, cells were treated with single concentrations of PHA665752 and in combination with canertinib in the presence of both growth factor concentrations 0.2 or 20 ng/mL. There was no significant difference in the cell numbers of OVCAR-5 clusters (**Figure 5.7 A, B**) and SKOV-3 compact aggregates (**Figure 5.7 E, F**) in the presence of 0.2 or 20 ng/mL of GF, with increased concentrations of PHA665752 alone, nor in combination with canertinib. However, there was a slight, but significant decrease in SKOV-3 cell numbers with the combination and the lower GF concentration. No effect on cellular metabolism was observed in both OVCAR-5 clusters (**Figure 5.7 C, D**) and SKOV-3 compact aggregates (**Figure 5.7 G, H**) with both growth factor concentrations. This suggests that for the most part the inhibitors are not compromising cell growth within the 4 hours timeframe, but rather are inhibiting adhesion.

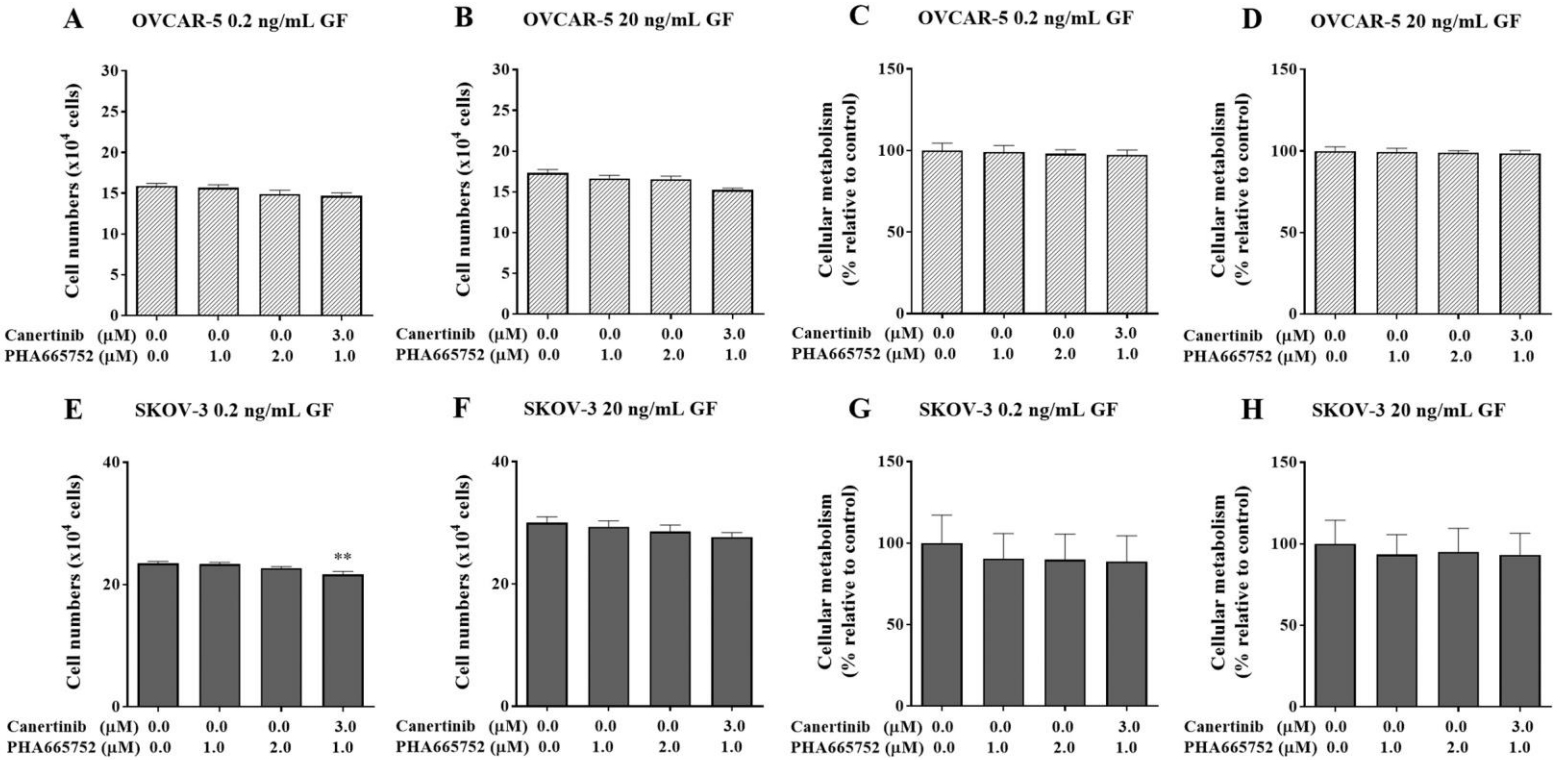

Figure 5.7. The effect of 4 hours incubation with different concentrations of PHA665752 alone or in combination with canertinib on the growth and metabolism of OVCAR-5 cellular clusters and SKOV-3 compact aggregates. Cell growth of OVCAR-5 (A and B) and SKOV-3 (E and F) exposed to single PHA665752 or in combination with canertinib in the presence of 0.2 or 20 ng/mL of GF. Metabolism of OVCAR-5 (C and D) and SKOV-3 (G and F) for the same conditions Data are expressed as means ± S.E.M (n=9). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.8 An RGD-containing peptide reduced adhesion.

The previous results suggest that the inhibitors compromise cellular adhesion to the collagen gel matrix. The integrin subunits β1 and β4 are transmembrane proteins that can form cell adhesions. They have previously been found to be associated with EGFR/HER-2 and c-MET and thus play a crucial role in the development and progression of ovarian cancer [372, 658].

To investigate the involvement of integrins in the adhesion process, clusters of OVCAR-5 and compact aggregates of SKOV-3 were exposed to RGDS-peptides both in the presence and absence of the combination treatment of canertinib and PHA665752. RGDS is a peptide that inhibits the binding of integrins to ECM proteins [366, 666]. Cells were maintained in serum free medium (SFM) supplemented with 0.2 or 20 ng/mL of GF and an adhesion assay was performed to determine the number of adherent cells. Control cells were treated with RGES which is an RGD-related peptide that serves as control for its inhibitory effects on fibrinogen binding. Aspartic acid is replaced with glutamic acid which abrogates the inhibitory effects on integrins and ECM proteins [667].

In the presence of 0.2 ng/mL GF, OVCAR-5 cellular clusters showed a concentration dependent decrease in cell adhesion with RGDS when compared to three types of controls: an acetate control (cells treated with acetate at the same concentrations used for RGDS to ensure that the inhibitory effect of RGDS is not affected by the pH changing acetate), and cells treated with RGES. The combination of canertinib and PHA665752 strongly inhibited adhesion as described earlier although the addition of RGDS to the combination did not reduce adhesion any further (**Figure 5.8 A**). Similar effects were observed in the presence of 20 ng/mL of GF (**Figure 5.8 B**).

SKOV-3 compact aggregates showed similar trends in the presence of 0.2 and 20 ng/mL of GF (**Figure 5.9 A, B**).

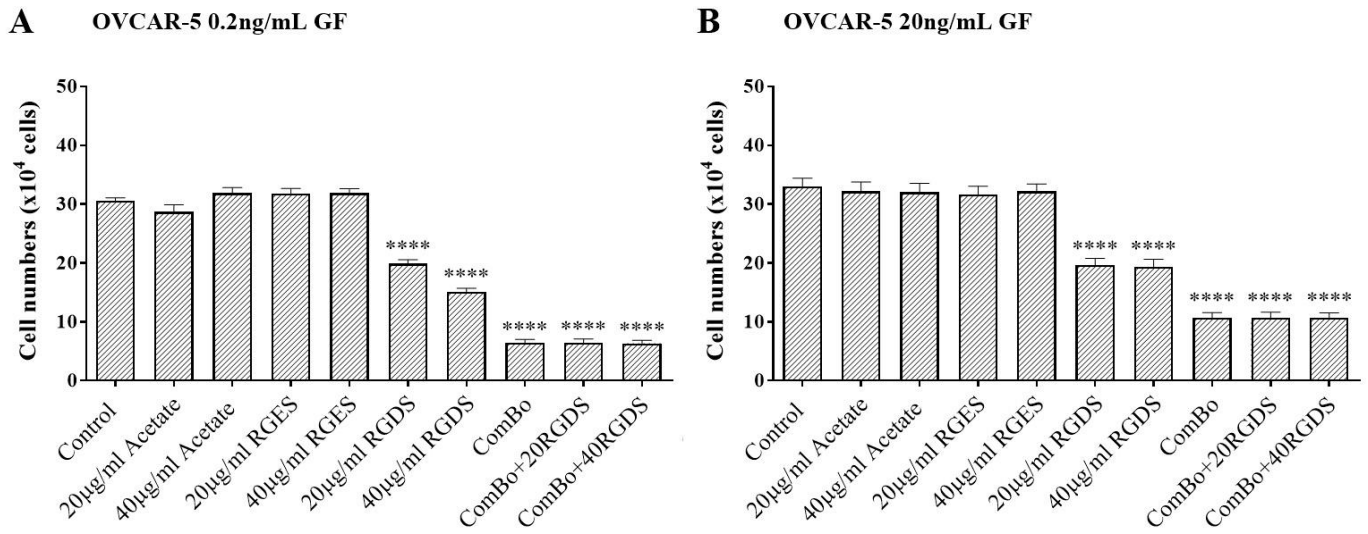

Figure 5.8. The effect of an RGD-containing peptide on cell adhesion in the presence of the inhibitors. OVCAR-5 cellular clusters in the presence of (A) 0.2 or (B) 20 ng/mL of GF. Cells were treated with 20 or 40 µM RGDS alone, or in combination with canertinib (3µM) and PHA665752 (1µM). Controls were comprised of untreated, acetate or RGES treated cells. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

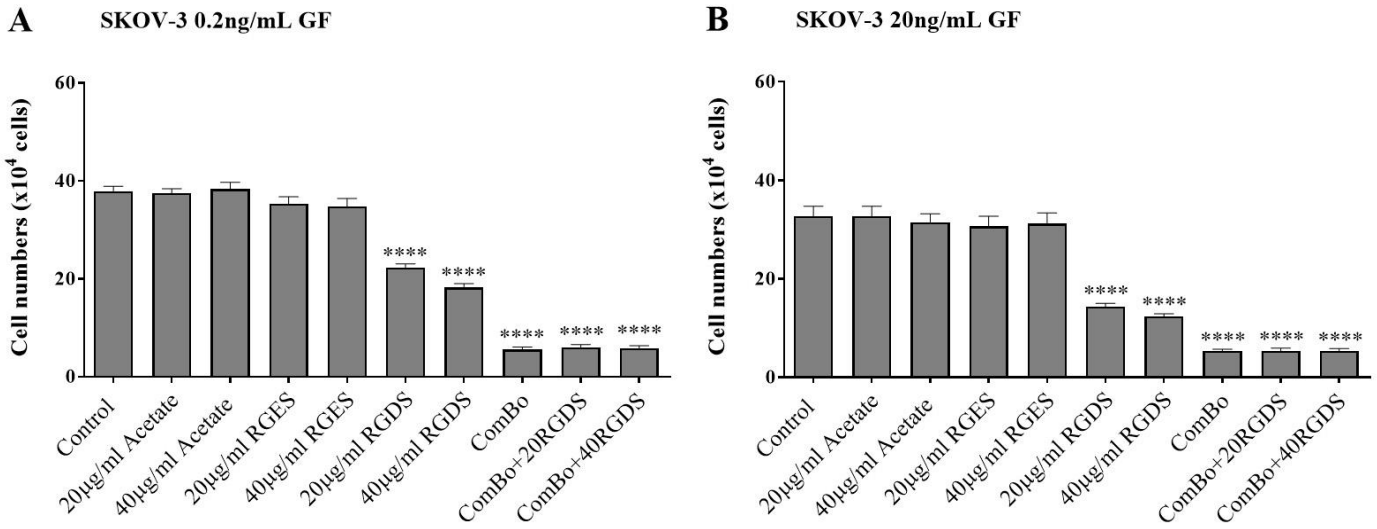

Figure 5.9. The effect of an RGD-containing peptide on cell adhesion in the presence of the inhibitors. SKOV-3 compact aggregates in the presence of (A) 0.2 or (B) 20 ng/mL of GF. Cells were treated with 20 or 40 µM RGDS alone, or in combination with canertinib (3µM) and PHA665752 (1µM). Controls were comprised of untreated, acetate or RGES treated cells. Data are expressed as means ± S.E.M (n=3). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control.

5.5.9 The effect of canertinib and PHA665752, singly and combined, on the total expression and phosphorylation of EGFR, HER-2, c-MET and the expression of β4 integrin subunits

The results above suggest that the cells are viable within the 4 hours incubation period with the inhibitors, but the adhesion of the cells was reduced. Therefore, following 4 hours of incubation with the inhibitors, an investigation of the total expression and phosphorylation of EGFR, HER-2, c-MET and then the expression of β4 integrin subunits was carried out using western blotting. The β1 integrin subunits were excluded from this part of the study because of difficulties in reliably obtaining bands for these on the western blots.

5.5.9.1 Effect on OVCAR-5 cellular clusters

In the presence of 0.2 ng/mL of GF, both canertinib and PHA665752, alone or in combination inhibited phosphorylation of EGFR (p-EGFR) in OVCAR-5 cells (**Figure 5.10 A**). Total expression of EGFR was slightly reduced with canertinib alone (**Figure 5.10 B**). PHA 665752 alone inhibited total expression and phosphorylation of MET protein (c-MET and p-MET) (**Figure 5.10 C and D**). The combination of inhibitors further decreased expression and phosphorylation. The expression of β4 was slightly reduced with the combination of the inhibitors, but not with the single treatments (**Figure 5.10 E**). HER-2 was undetectable in the OVCAR-5 clusters. Similar results were obtained with the presence of 20 ng/mL of GF (**Figure 5.10 F, G, H, I and J**).

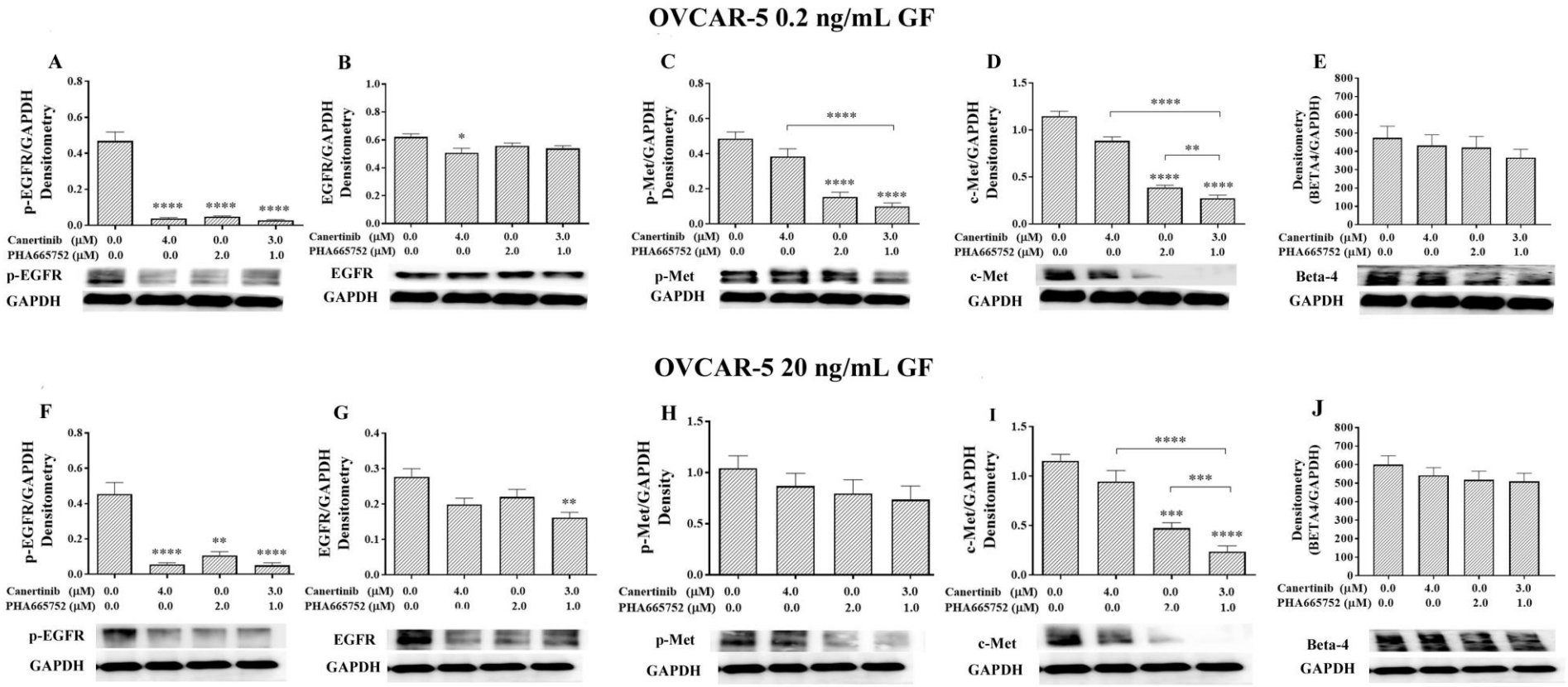

Figure 5.10. Densitometry index of OVCAR-5 cellular clusters showing the effect of canertinib and PHA665752 alone or in combination in the presence of 0.2 or 20 ng/mL of GF. Total expression and phosphorylation of EGFR (A, B, F and G) and c-Met (C, D, H, and I). Expression of β4 integrin subunits (E and J). Densitometry values are expressed as means ± S.E.M (n=9). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.9.2 Effect on SKOV-3 compact aggregates

In the presence of 0.2 or 20 ng/mL of GF, canertinib alone or in combination with PHA665752, inhibited total expression and the level of phosphorylation of EGFR (p-EGFR) (**Figure 5.11 A, B, H and I**). In contrast, in the presence of PHA665752 there were no significant changes. Similar trends were observed with the total expression and phosphorylation of HER-2 (**Figure 5.11 C, D, J and K**). Both inhibitors, alone and in combination, reduced the total expression and phosphorylation of c-MET (**Figure 5.11 E, F, L and M**). There was no reduction in the expression of β4 integrin subunits with any of the treatments (**Figure 5.11 G**).

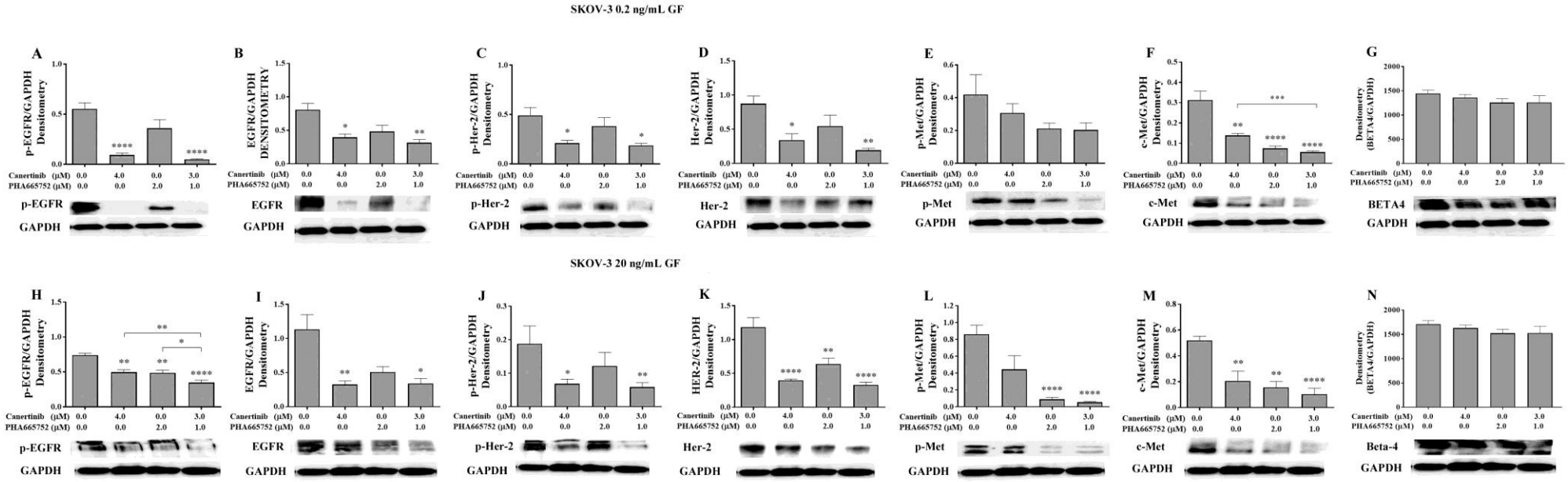

Figure 5.11. Densitometry index of SKOV-3 compact aggregates showing the effect of canertinib and PHA665752 alone or in combinationin the presence 0.2 or 20 ng/mL of GF . Total expression and phosphorylation of EGFR (A, B, H and I), Her-2 (C, D, J and K), and c-Met (E, F, L and M). Expression of β4 integrin subunits (G and N). Densitometry values are expressed as means ± S.E.M (n=9). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control or the other concentrations.

5.5.10 Immuno fluorescent imaging showed no changes in the expression of β1 integrin subunits in response to canertinib and PHA665752 alone or in combination

To investigate the effects of canertinib and PHA665752 on the expression of the β1 integrin subunit immunofluorescent staining was used. Clusters of OVCAR-5 cells and compact aggregates of SKOV-3 cells were treated with single inhibitors or a combination of inhibitors. Immunofluorescent imaging of β1 integrin subunits indicate that no observable changes occurred in the expression of the subunits, with either the single or combined treatments of the inhibitors, in both OVCAR-5 clusters and SKOV-3 compact aggregates (**Figure 5.12).**

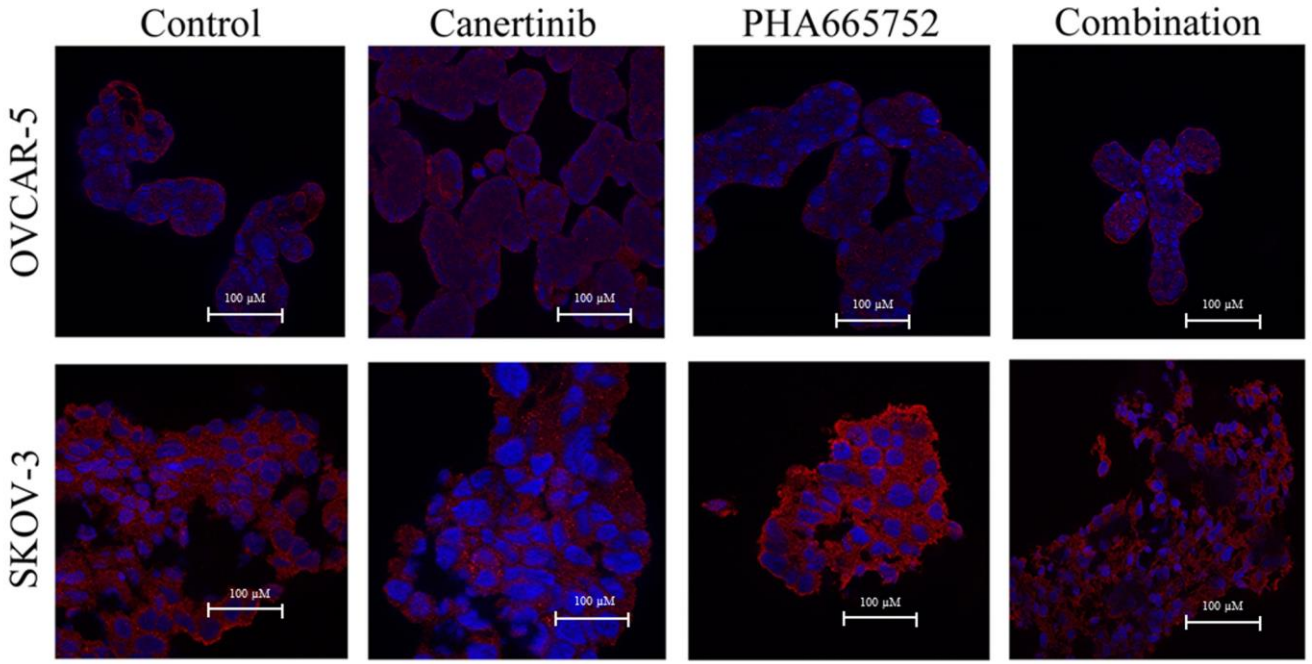

Figure 5.12. Immuno fluorescent imaging of OVCAR-5 cellular clusters and SKOV-3 compact aggregates showing the expressions of β1 integrin subunits in response to treatments with single or combined inhibitors. Blue colour are DAPI stained nuclei and the red colour are the plasma membrane associated β1 receptors.

5.5.11 Immuno fluorescent imaging show no changes in the expression of β4 integrin subunits in response to canertinib and PHA665752 alone or in combination

To investigate the effect of the inhibitors on the expression of β4 integrin subunits in the aggregates and clusters were stained with antibodies raised against the β4 subunit. Immunofluorescent images of β4 integrin subunits show no observable changes in the expression of the subunits in the presence of single or combined treatments with the inhibitors, in both OVCAR-5 clusters and SKOV-3 compact aggregates (**Figure 5.13**). The subunits appear to be mostly localised around the outside edges of the cell membranes.

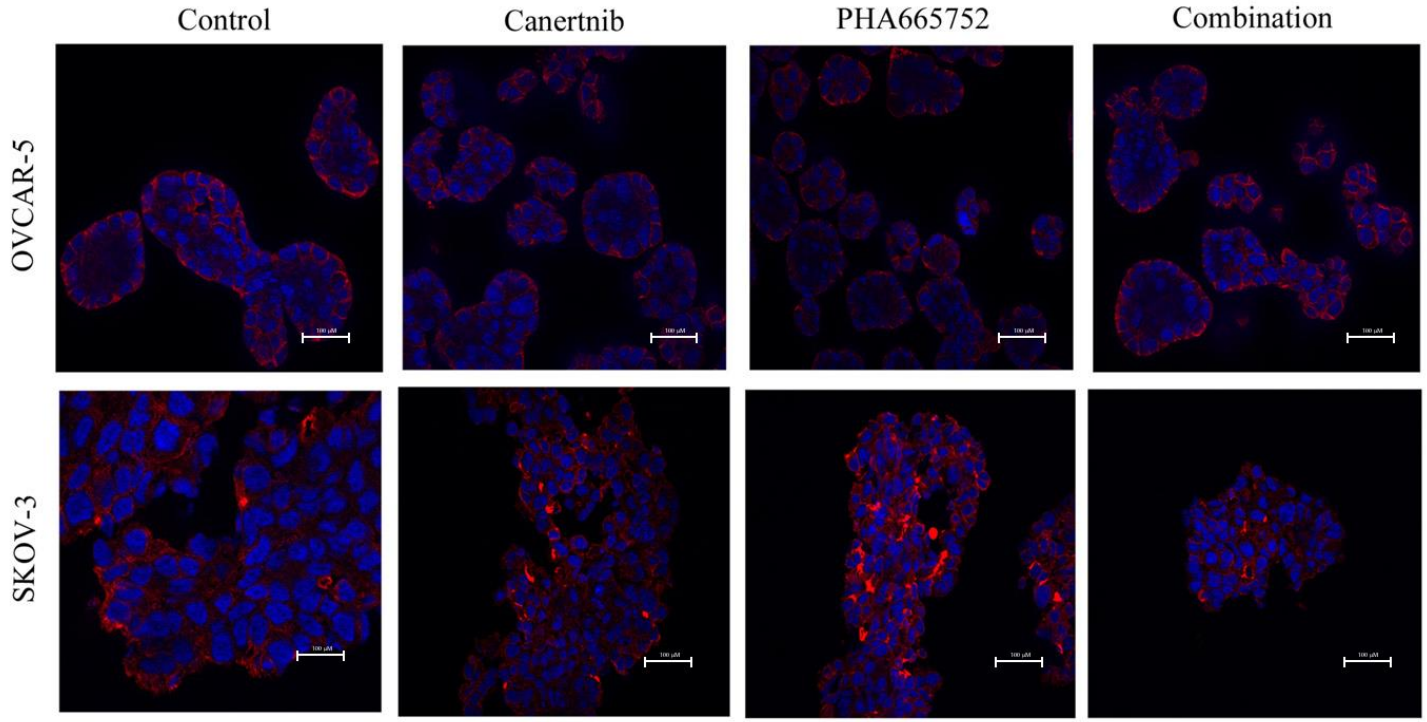

Figure 5.13. Immuno fluorescent imaging of OVCAR-5 cellular clusters and SKOV-3 compact aggregates showing the expressions of β4 integrin subunits in response to treatments with single or combined inhibitors. Blue colour are DAPI stained nuclei and the red colour are the plasma membrane associated β4 receptors.

5.5.12 Co-localisation of EGFR and HER-2 with β4 integrin subunits

The previous results suggest that the expression and phosphorylation of EGFR and HER-2 were reduced with single or combined inhibitor treatments resulting in a loss of cellular adhesion. However, the expression of β4 integrin subunits was unchanged. It was possible that the loss of adhesion might be due to a compromisation of the physical interaction between EGFR and HER-2 with β4 integrin subunits. To investigate this, the co-localisation of these receptors was studied using immunofluorescence. Cells were stained with antibodies for EGFR, HER-2 and β4 integrin subunits. There was a significant reduction in the co-localisation of EGFR/β4 and HER-2/ β4 in both of OVCAR-5 cellular clusters (**Figure 5.14 A**) and SKOV-3 compact aggregates (**Figure 5.14 B** and **C**). This may explain the observed reduction in cell adhesion without any observable change in the expression of integrin subunits.

The fluorescent intensity of the receptors was also analysed. There was a significant reduction in EGFR intensity in OVCAR-5 cellular clusters, while no significant reduction in the intensity of staining of β4 integrin subunits was observed (**Figure 5.15 A**). Similar results were obtained with SKOV-3 compact aggregates with anti- EGFR, HER-2 and β4 antibodies (**Figure 5.15 B and C**).

The co-localisation immunofluorescent images of EGFR and the β4 subunit for OVCAR-5 cellular clusters with single and the combination treatment of inhibitors is shown in **Figure 5.16**. **Figures.5.17. and 5.18** illustrate the immunofluorescent images of SKOV-3 compact aggregates showing the co-localisation of EGFR and HER-2 with β4 subunits respectively. These results are consistent with the previous results obtained from Western blotting analysis.

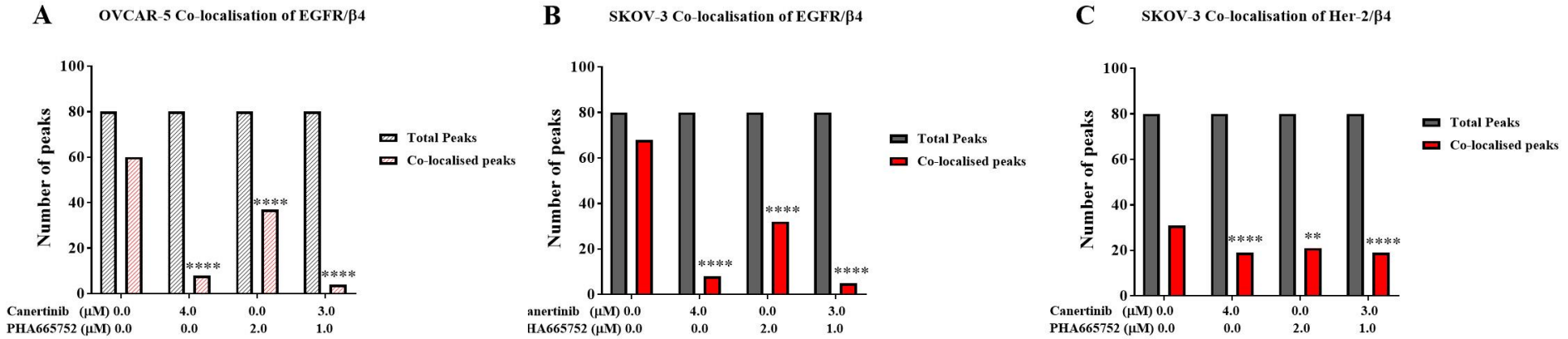

Figure 5.14. Co-localisation of EGFR and Her-2 with β4 in OVCAR-5 cellular clusters and SKOV-3 compact aggregates treated with single or combination of the inhibitors. (A and B) EGFR/β4 for OVCAR-5 and SKOV-3; (C) Her-2/β4 for SKOV-3. The total peaks of each receptor and their co-localisation were counted by analysisng immunofluorescent images with ZEN programme (Carl-Zeiss). Data are expressed as means ± S.E.M (n=9).Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control

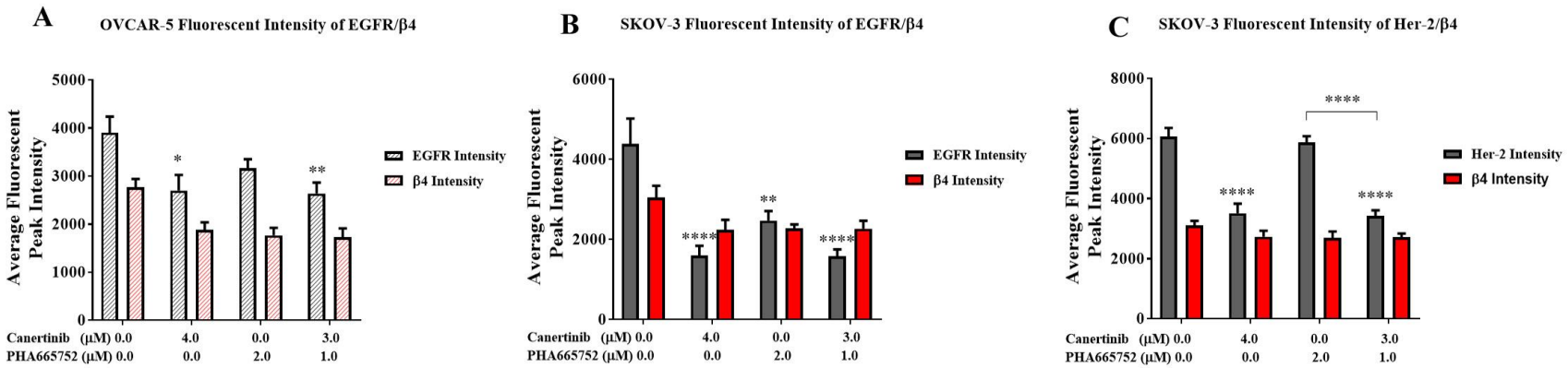

Figure 5.15. Receptor intensities from immunofluorescent immages of OVCAR-5 clusters and SKOV-3 compact aggregates treated with single or combined inhibitors. The intensity of the peaks of EGFR/β4 in OVCAR-5 and SKOV-3 (A and B respectively) and Her-2/β4 in SKOV-3 (C) were recorded and analysed for significance. The peaks intensity was obtained by analysing immunofluorescent images with ZEN programme (Carl Zeiss). Data are expressed as means ± S.E.M (n=9). Data considered statistically significant are indicated as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****) compared to the control

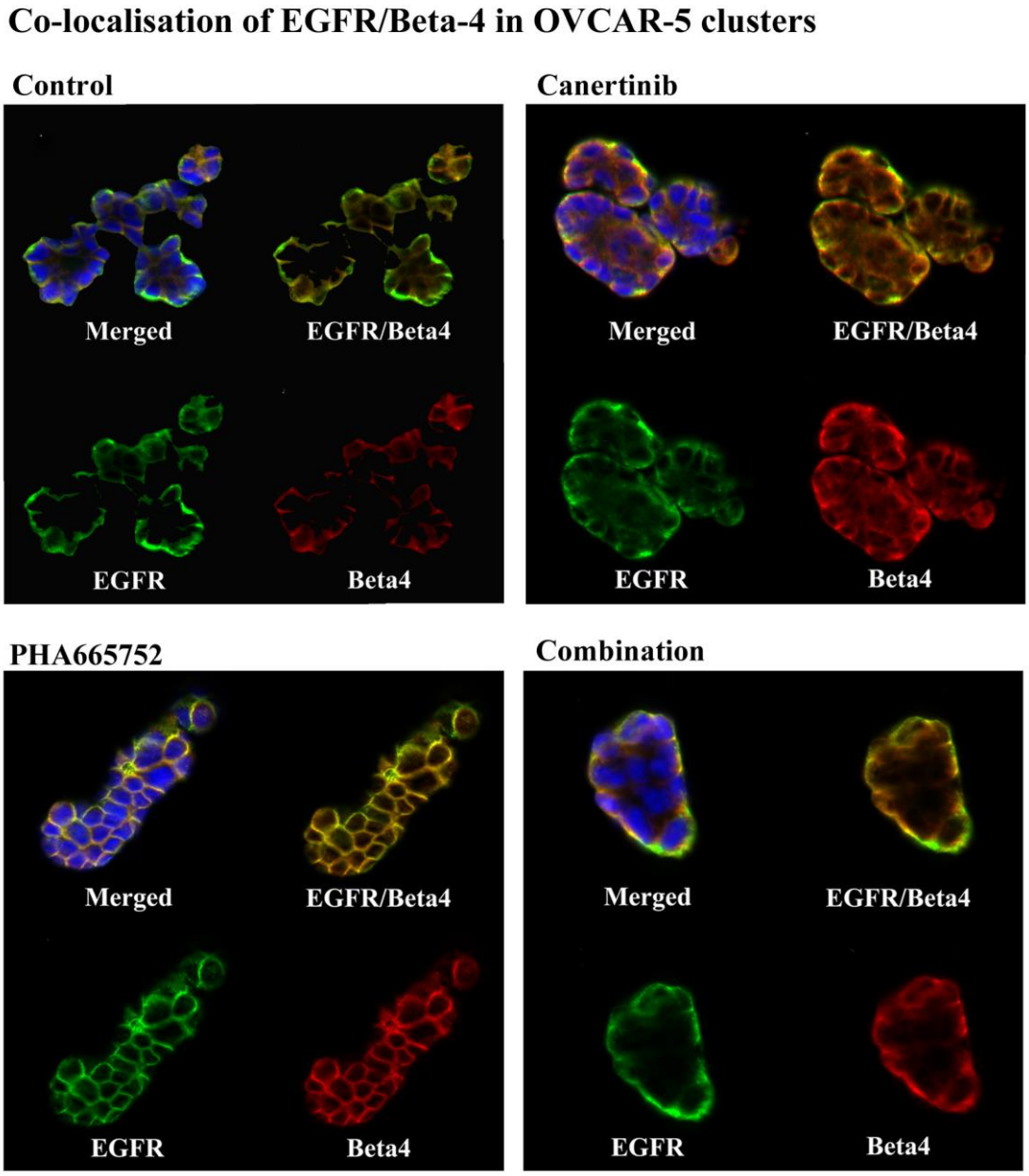

Figure 5.16. Immunofluorescent images of OVCAR-5 cellular clusters treated with single or combination of canertinib and PHA665752 showing co-localisation of EGFR/β4. Blue colour are DAPI stained nuclei, red colour are the plasma membrane associated β4 receptors, and green colour are EGFR.

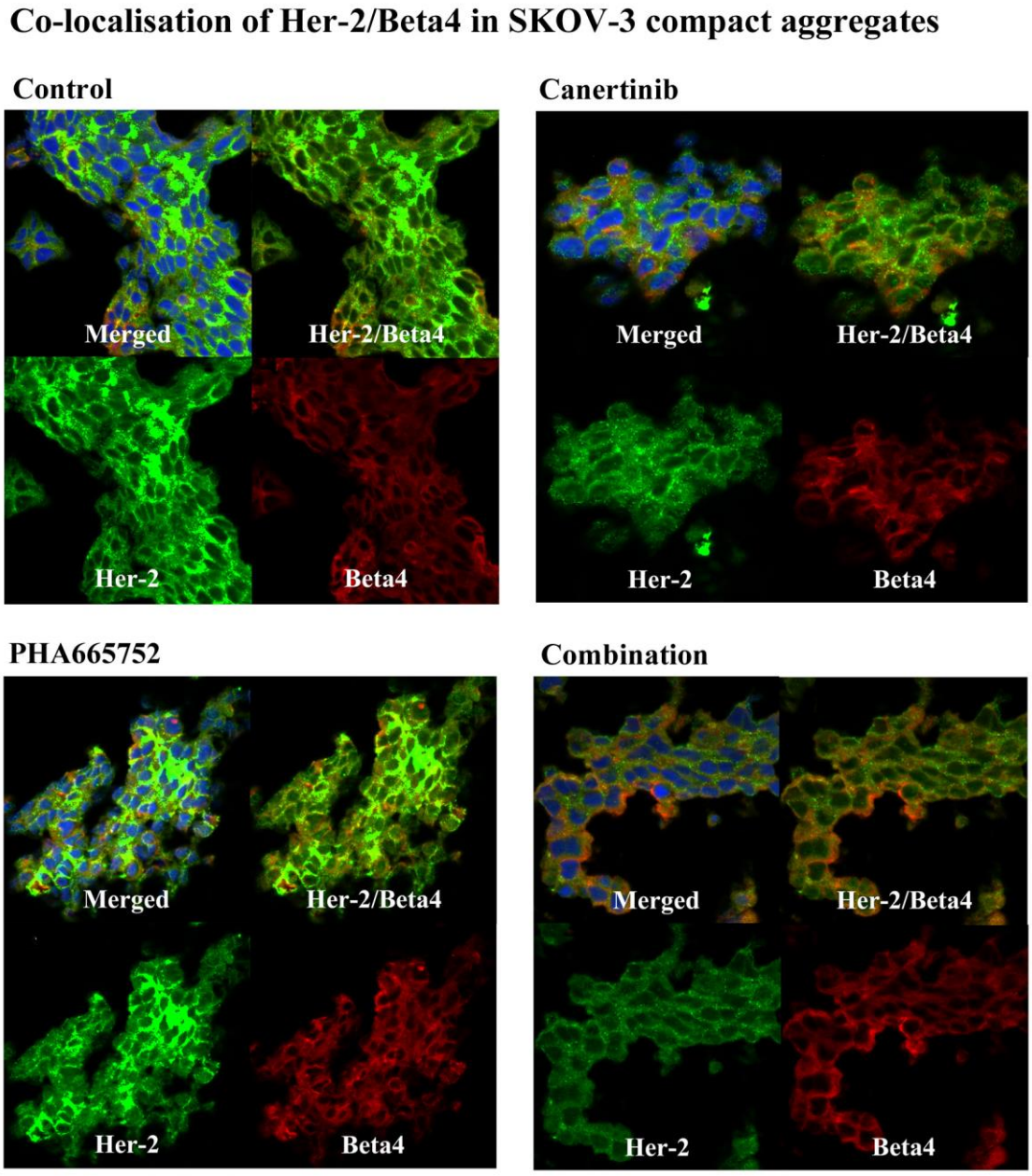

Figure 5.18. Immunofluorescent images of SKOV-3 compact aggregates treated with single or combination of canertinib and PHA665752 showing co-localisation of Her-2/β4. Blue colour are DAPI stained nuclei, red colour are the plasma membrane associated β4 receptors, and green colour are Her-2.

5.6 Discussion

Understanding the stages of ovarian cancer metastasis is important for identifying potential targets for cancer treatments. The spread of malignant cells into the peritoneum is a complicated multistep process that leads to cancer progression, and it involves many mechanisms and interactions between the malignant cells and the ECM. The ability of ovarian cancer cells to adhere to the ECM and mesothelial lining of the abdominal cavity is a unique characteristic of the malignant cells [616, 668] that exfoliate from the primary tumour and shed into the peritoneal fluid [669]. In the peritoneum, free floating malignant cells are found as single cells or forming clusters and aggregates [33, 157, 638]; hence studies that focus on multicellular *in vitro* responses may better mimic the *in vivo* microenvironment [364].

The ECM components, including fibronectin, laminin and type I and IV collagens [670, 671] control a variety of cellular activities in normal and malignant cells including growth, proliferation and differentiation. This is achieved through their binding of integrins [364, 616, 672, 673]. Integrins are cell surface receptors for ligands in the ECM [674], responsible for regulating cell-cell and cell-ECM interactions. They facilitate adhesion, migration and the invasion of tumour cells into other organs and angiogenesis [675, 676]. In addition to their role as ECM organisers [677], integrins have been shown to regulate cell adhesion to ECM components [653, 678, 679] and facilitate the motility of ovarian carcinoma cells [652, 680, 681]. The presence of integrins in malignant cells is an indicator of a poor prognosis and the spread of the disease to other locations in the body [9, 669, 682]. Integrin β1 and β4 subunits have been suggested to be key drivers in the progression of ovarian cancer, particularly in the adhesion of the cells to fibronectin, laminin and type I collagen [668, 670, 683]. There is a well-established association between those integrin subunits and the receptor tyrosine kinases, EGFR, HER-2 and c MET that have been the focus of earlier parts of this thesis. Several studies suggest that the cross talk between these receptors is a key driver in metastasis and in angiogenesis in breast and ovarian tumours [372, 376, 658, 684, 685].

The main objective of this chapter was to evaluate the effect of two tyrosine kinase inhibitors, canertinib and PHA665752 on cellular adhesion of OVCAR-5 and SKOV-3 ovarian cancer cell lines in the presence of EGF and HGF. These cell lines have been found to possess the ability to form multi cellular clusters and aggregates that resemble the *in vivo*

ovarian carcinoma spheroids formed in the ascites [686]. The ability of these cell lines to adhere to mesothelial cells has also been reported in an *in vitro* model [687, 688]. The major finding of the work presented in this chapter is that these inhibitors compromised cellular adhesion, via inhibition of the targeted receptors. The objective was to expose OVCAR-5 cellular clusters and SKOV-3 compact aggregates to the inhibitors for a specified period of time that was sufficient to allow the cells to adhere to the collagen-gel matrix without compromising their cellular viability. Various time periods to facilitate cell adhesion have been used in other studies [528, 653, 668, 671]. A time frame of 4 hours incubation has been suggested to be optimal for cellular adhesion of OVCAR-5 cells [364] and therefore this was used in this study. Additionally, an incubation period with the inhibitors prior to the adhesion assay was also chosen so that the cell viability was not compromised [542].

The data presented suggest that OVCAR-5 cellular clusters and SKOV-3 compact aggregates retain high expression of β1 and β4 integrin subunits and that these facilitate the adherence of these cells to the matrix. This is consistent with other studies showing the high expression of these integrin subunits in ovarian cancer cells [689]. Other studies have shown that β1 and β4 integrin subunits are highly expressed in epithelial tumours of breast and colorectal in addition to ovarian carcinomas and that these play a crucial role in cell motility and metastasis [690-693]. The SKOV-3 cell line has also been shown to exhibit high invasive behaviour by utilising β4 integrin subunits [694, 695].

As mentioned earlier, the *in vivo* microenvironment is rich with growth promoting factors that play a pivotal role in modulating cell adhesion [696-699]. Of particular interest in this study are EGF and HGF which are the specific ligands for the EGFR/HER-2 and c-MET receptors that have shown to synergistically modulate the integrin-induced adhesion of ovarian cancer cells [526]. Hence the effect of these growth factors on cell adhesion was investigated.

The data presented indicates a concentration dependant increase in cell adhesion with either of the growth factors applied alone or in combination (GF). This is consistent with the previous data from this study, showing the growth promoting properties of EGF and HGF on ovarian cancer clusters and aggregates [596]. These results are also in line with other reports

indicating a role for EGF and HGF in promoting adhesion, migration and survival of malignant tumour cells, particularly ovarian cancer cells [278, 526, 528].

Small molecule tyrosine kinase inhibitors have been suggested to inhibit cell growth, motility, adhesion and invasion of advanced ovarian cancer cells [335, 345, 525, 528, 664, 665]. Moran and colleagues demonstrated that INC280, a small molecule, ATP competitive c-MET inhibitor, reduced migration and HGF-induced adhesion in SKOV-3, OVCAR-3, and CAOV3 ovarian cancer cell lines [528].

As described earlier in this thesis, canertinib and PHA665752 have inhibitory effects on GF-induced cell growth and metabolism of OVCAR-5 clusters and SKOV-3 compact aggregates [596]. To identify any anti-adhesion effects of the inhibitors, the impact on GF-induced cell adhesion to a collagen gel matrix was investigated. Canertinib inhibited adhesion of both cell lines in a dose dependant manner. To the best of my knowledge this is first work that demonstrates an inhibitory effect of canertinib alone and in combination with PHA665752 on cell adhesion. This inhibition was not affected by the concentration of the added growth factors. Although the presence of growth factors was essential for the activation of the receptors and thus the response to the drugs, there was no additive inhibition with higher growth factor concentrations. This is consistent with previous data from chapter 1 which suggests that a low concentration of 0.2 ng/mL of GF (the growth factor mixture) was sufficient enough to activate the receptors and to elicit a cellular response to the treatments. The second inhibitor PHA665752 also exhibited a concentration dependent inhibition of cell adhesion in both cell lines. This is consistent with previous reports of the inhibitory properties of PHA665752 on HGF-mediated adhesion of myeloma cells to fibronectin [700].

The combination of both inhibitors elicited an additive effect and cellular adhesion was lower than that observed with single inhibitors alone. Again to the best of my knowledge there have been no previous reports that demonstrate the combined effects of canertinib and PHA665752 on GF-induced cellular adhesion of OVCAR-5 clusters and SKOV-3 compact aggregates

As reported in previous chapters, the inhibitors were effective in inducing cell death after an incubation period of 48 hours during which cell number and cellular metabolism were significantly decreased. In the adhesion experiments no reduction in cell number or cellular

metabolism took place when the cells were exposed to the inhibitors for a total of eight hours (i.e. the 4 hours of exposure to the inhibitors was followed by another 4 hours of the adhesion assay). The suggested time frame to facilitate adhesion ranges from 30 minutes to 24 hours [528, 671, 695, 701-703]. Therefore, the reported loss of cell adhesion is unlikely to be due to cell death as the cell numbers and cellular metabolism were not affected by the 4 hours of exposure to the inhibitors.

In order to address the mechanisms by which inhibition of tyrosine kinase signalling could affect the cellular adhesion to the collagen matrix, cells were exposed to RGDS alone or in combination with the inhibitors. Integrins bind to proteins of the ECM at an RGD containing motif and thus the inclusion of RGD peptides in culture media has commonly been used to inhibit cell adhesion [704]. RGDS at a concentration of 40 μM decreased cell adhesion, which would suggest the importance of integrins in adhesion. This finding is supported by other studies demonstrating that β1 integrins subunits play a role in the adhesion of ovarian cancer cells [364, 652, 705]. When canertinib and PHA665752 were included the reduction in adhesion was not enhanced further by the inclusion of the RGDS. While it could be predicted that the RGDS would reduce adhesion further it should be noted that the tyrosine kinase inhibitors had already elicited a very significant reduction in numbers of adherent cells. Those cells that remained adherent may represent a small subpopulation that are not affected by the RGDS. These may express other adhesion molecules or alternately integrin subunits that are less sensitive to the RGDS. Clearly this is an area that warrants further investigation but unfortunately due to time constraints this was not investigated further in the current study.

There is evidence to suggest that there may be a co-activation of downstream signalling molecules of the integrins and growth factor receptors that regulate proliferation, migration and survival [706, 707] in normal and malignant cells. This co-operation is thought to regulate cell cycle responses [646, 708]. Gambaletta and colleagues suggested a mechanism by which β4 integrin subunits co-operate to promote tumour invasion of NIH373 cells in an *in vitro* model study [373]. They showed that expression of both HER-2 and α6β4 integrins is required for enhancing of PI3K-dependent invasion through matrigel. They suggested a specific region on the cytoplasmic tale of β4 subunits that is pivotal for the integrins' ability to enhance invasion. It was suggested that neither the extracellular domain of the β4 subunit nor its hetero-dimerisation with the α6 subunit contribute to any enhancement of invasion and

that the cooperation of the β4 subunit with HER-2 is essential for this matter. This is also consistent with other reports indicating the ability of β4 subunits to enhance invasion independently of dimerisation with α6 subunits [656, 709].

Cross talk between integrins and RTKs has also been shown to result in regulation of the activities of RTK [710]. Furthermore, it has been suggested that the phosphorylation of several RTKs, including EGFR, may be stimulated by integrin signalling [711] in the absence of growth factor ligands. The integrin-dependent activation of EGFR subsequently activates downstream signalling molecules of growth pathways ERK1/2, MAPK and Akt [371]. On the basis of these reports, an analysis of the total expression and phosphorylation of EGFR/HER-2 and c-MET and β4 subunit expression was studied. There was a down regulation of EGFR/HER-2 and c-MET but not β4 subunits upon exposure to the inhibitors and this may have affected the cross talk between the various receptors and have accounted for the loss of adhesion. However, this does not explain why cell adhesion was lost independent from β4 expression, which was not affected by the inhibitors. A possible explanation is that these integrin subunits are phosphorylated upon activation of EGFR. This is based on the idea that certain integrins may have affinity for certain RTK i.e., the integrin β1 subunit is associated with EGFR [712-714] and the integrin β4 subunit is associated with EGFR [715], HER-2 [370] and c-MET [375]. The association of β4 subunits with EGFR/HER-2 and c-MET, along with the above results could suggest that the downregulation of the receptors inhibited the adhesion through the inhibition of shared downstream signalling molecules, for example ERK and Akt. This possibility is consistent with the results presented in the previous chapter where there was a downregulation of total expression and/or phosphorylation of Akt and ERK in the presence of canertinib and PHA665752 individually or in combination. Moreover, this inhibition may lead to a decrease in the co-localisation of β4 and EGFR/HER-2. This latter possibility was further supported by the findings that both canertinib and PHA665752, alone or in combination, were able to inhibit the co-localisation of β4 integrin subunits and EGFR/HER-2. The co-localisation is due to a physical interaction at cell-cell contact sites as demonstrated by Yu et.al.[714].

To the best of my knowledge, no previous studies have reported the effect of combining canertinib and PHA665752 on cell adhesion. The results suggest that although integrins are considered key drivers in the adhesion of ovarian cancer cells, inhibition of EGFR/HER-2 and

c-MET could potentially affect the downstream co-localisation of the receptors with integrin subunits and therefore inhibit cell adhesion. The co-localisation of EGFR and β4 was observed by immunofluorescent microscopy. This is consistent with other studies confirming the co-localisation of integrins with EGFR using immunofluorescent microscopy [714].

In summary, the treatment of OVCAR-5 cellular clusters and SKOV-3 compact aggregates with canertinib and PHA665752 as single agent or in combination compromised cell adhesion and this may occur via the inhibition of downstream signalling molecules, thus affecting the intracellular co-localisation of the receptors. Further work is required to explore the effect of the two small molecule inhibitors on the co-localisation of β4 integrin subunits with c-MET and the β1 integrin subunits possible co-localisation with EGFR, HER-2 and c-MET.

CHAPTER SIX

SUMMARY

Two of the hallmarks of cancer are uncontrolled, sustained cellular proliferation [22, 31, 220] and the evasion of growth suppressors. Ovarian cancer is the eighth most common cause of cancer related deaths in women worldwide [716]. Almost 75% of ovarian cancer patients respond to chemotherapy [7], but due to a typically late diagnosis where approximately 75% of patients present with an advanced stage [7] there are high rates of recurrence of the disease and consequently high mortality rates [13, 14]. Like many cancers, ovarian cancer is a complex disease, in part due to the diverse tissues and different cell types forming the ovaries [717]. Consequently, multiple types of ovarian cancer can arise.

In common with other cancers such as breast, colon, liver and gastric cancer, ovarian cancer can arise due to the elevated expression and activation of receptor tyrosine kinases. Elevated expression of these receptors can be linked not just to cancer development, but also to a poor prognosis with disease spread, resistance to chemotherapy and low 5 year survival rates [305, 334, 465, 468, 718]. The receptor family comprises members that regulate signal transduction pathways that are key drivers in cell survival [719].

Inhibitors that target EGFR and HER-2 which are members of the ErbB family of receptor tyrosine kinases have been used successfully in the treatment of breast, colon, gastric and NSCLC cancer [241, 462, 531] and their combination with other types of chemotherapy can have good outcomes [547, 550]. However, these treatments have not been as successful with advanced ovarian cancer and despite a reduction of tumour burden, the rate of disease recurrence can still be high [547]. Another tyrosine kinase receptor that is of interest is c-MET [537, 720, 721]. which can be abnormally expressed in cancer cells of epithelial origin [721]. Its specific ligand is HGF which is present at high levels in the plural fluids and tissues surrounding tumours [486, 487]. It has been suggested that malignant cells from NSCLC and Head & Neck carcinomas may utilise c-MET as an alternative route to activate growth signalling pathways if EGFR and HER-2 are inhibited and hence the cells can surpass attempts to stop their growth [720, 722]. The research described in this thesis was an attempt to simultaneously inhibit EGFR/HER-2 and c-MET and observe the effects on two ovarian cancer cell lines.

In Chapter-3 the aim was to investigate the effects of two receptor tyrosine kinase inhibitors, canertinib and PHA665752 on the growth, metabolism and proliferation of the OVCAR-5 and SKOV-3 ovarian cancer cell lines. Following this their effects on downstream signalling molecules were investigated. These experiments were carried out in the presence of the growth factors that bind these receptors i.e. epidermal growth factor (EGF, which binds EGFR) and hepatocyte growth factor (HGF which binds c-MET) which both were used at two different concentrations. The low concentration was 0.2 ng/mL GF, which is the approximate level in ovarian cancer patients [486, 487, 500] and the higher concentration was 20 ng/mL GF, a concentration which has frequently been used in laboratory studies [278, 435, 496].

The cell lines were grown as 3D clusters and aggregates in an attempt to better represent their normal microenvironment where they exist as single cells or floating aggregates. This could be a better representation than the 2D monolayer cell culture where cell shape, cell-cell and cell-ECM interactions may be altered due to the growth on a flat plastic surface [454, 723-725]. Studies have also shown that cells in 3D aggregates show gene expression profiles similar to those of *in vivo* cells, which is in contrast to those in a 2D monolayer [726]. The response of cells to treatments when in 3D culture may thus be more clinically relevant [727]. Furthermore, cells in the multi cellular aggregates are likely to go through similar stages/conditions as tumour cells *in vivo* such as being normoxic, hypoxic, quiescent, necrotic and/or dying [728]. Collectively, the use of 3D cellular aggregates appears to be a more biologically relevant model for this study.

The inhibitors affected growth of the 3D cell cultures and the expression and phosphorylation of downstream signalling molecules that are part of the intracellular survival pathways. The expression of the cell proliferation marker PCNA was also reduced. The combined effect of these two inhibitors was more potent than each inhibitor alone and suggests that blocking dual receptors may induce a greater impact on cellular growth and survival. Additionally, cells showed lower metabolic activity in the presence of the inhibitors alone or with the combined treatment. VEGF secretion was also decreased upon treatments with the inhibitors.

The cellular response to the inhibitors was less pronounced in the presence of 5% FBS. Cell growth decreased but this was not as marked as in the presence of the growth factors

Furthermore cellular metabolism was not affected. A possible explanation for this could be that FBS is rich in growth promoting factors that may contribute to the cellular resistance to the inhibitors. Further experiments investigating the effect of different concentrations of the FBS would be of interest

The results of chapter 3 raise the possibility that combining small molecule inhibitors that block different receptors may be a potential treatment option for ovarian cancer. They also suggest an ability of ovarian cancer cells to utilise multiple pathways in order to survive apoptotic factors induced by the blockage of one main growth pathway [241, 334, 338]. To the best of my knowledge, this is the first time that the combination of these inhibitors has been studied in ovarian cancer 3D clusters.

One limitation in this study is that only two ovarian cancer cell lines were investigated due to time and resource constraints. Further study of more ovarian cancer cell lines with differential expression of the RTKs could enhance our knowledge of the inhibitors actions. It would also be interesting to explore the effect of canertinib and PHA665752 on other downstream signalling pathways important for maintain the hallmarks of ovarian cancer cells such as JAK/STAT, PI3K and NFkB [729-733]. It would be of interest to study the effects of the combination on primary tissue from ovarian cancer patients and to *in vivo* preclinical studies using mouse models or chicken embryos.

As mentioned above, ovarian cancer cells in the body are typically present as floating single cells or cellular aggregates. Malignant ascites is found in at least 37% of advanced ovarian cancer patients and this has been recognised as a poor prognostic factor in advanced ovarian cancer [165, 176, 574]. Ascitic fluid may be important in regulating the cell signalling processes of the malignant cells once they have shed into the abdominal cavity [626].The increased expression of growth receptors in cells floating in malignant ascites suggests that soluble factors in the fluid may then be able to enhance cell growth, migration [486] and resistance to chemotherapy [613, 734]. This raises the question of what effect ascitic fluid may have on the efficacy of the drugs that was described in chapter 3 and formed the basis of the results that were presented in chapter 4.

The results in chapter 4 demonstrate that the ascitic fluid can have a significant effect in reducing the efficacy of canertinib and PHA665752 and suggests that components in the

ascitic fluid could lead to the resistance and survival of ovarian cancer cells in the ascitic fluid. These results were consistent with Mo and colleagues suggesting that ascites increases cancer cell chemo-resistance [180]. They showed that the treatment of murine ovarian cancer cells with paclitaxel (a first line chemotherapeutic agent) in the presence of ascitic fluid made cells less sensitive to the drug [180].

One possibility explanation of these results is that the presence of ascitic fluids affects the uptake of the drugs or that there was increased efflux of the drugs. In addition to inducing the production of ascites, the presence of malignant ovarian tumours may also cause increased vascular permeability [619]. This may increase the protein content of the ascitic fluid and in particular human serum albumin [170, 735]. There have been reports that tyrosine kinase inhibitors can bind to albumin [602]. This would be consistent with the reported effects of BSA in the current thesis and thus this may prevent the drugs from entering the cells. Note that no such effects were observed with FBS in chapter 3 but the concentrations of albumin in that particular assay were much lower than the likely albumin levels in ascitic fluid. The serum albumin may not be the only factor affecting the uptake of the drug into the cells. Uptake was significantly lower in the ascitic fluid treated cells compared to the BSA treated cells and so it is likely that there may be other factors in the ascitic fluid that can influence drug uptake.

It should be acknowledged that while the suggestion is made that the albumin may be limiting uptake of the PHA665752 there is no direct evidence to suggest that this may be the case. It is also unclear what effects the ascitic fluid may have on the uptake of canertinib. Experiments where albumin is removed from the ascitic fluid before the addition of inhibitors may give a clearer view of its effect on the efficacy of the drugs. Some researchers have suggested boiling the ascitic fluid in order to denature the protein contents [162] but this would effectively eliminate all proteins not just the albumin. Unfortunately the albumin concentration in the ascitic fluids was not determined in this project due to time constraints. This is something that a follow on study could investigate.

Ascitic fluid from 3 patients was tested in the current thesis and it is notable that the reported effects were consistent irrespective of which patient the fluids were from. It would be of interest however to test the ascitic fluids from more patients. The clinical records of the three

patients from whom the fluids were obtained revealed that all three patients were from the same molecular subtype (see appendix). Future studies could investigate the effects of fluids from patients with different subtypes of ovarian cancer. This could be coupled with studies involving additional ovarian cancer cell lines with distinct molecular backgrounds.

Cell growth was significantly higher in the ascitic fluid, consistent with the observed increased expression of PCNA. Furthermore the ascitic fluid decreased the expression levels and activation of the receptors. This was also the case for the downstream protein, ERK. While the receptors were downregulated, the total expression and phosphorylation of Akt was significantly increased. Akt is part of the PI3K/Akt signalling pathway downstream of EGFR/HER-2 and c-MET and can regulate cell proliferation, invasion and angiogenesis [733]. Other studies have shown that Akt is elevated in the presence of ascitic fluid which may explain the enhanced proliferative properties of the cells [156, 618]. This is also in line with the studies indicating that ascites is rich with growth promoting factors that may augment cellular survival through other signalling pathways separate from EGFR/HER-2 and c-MET [736].

The cellular aggregates floating in the ascitic fluid have the ability to adhere to the mesothelial lining of the abdominal cavity, to proliferate there and to generate larger tumour nodules. This is an important stage in dissemination of cancer cells to other locations in the body. Therefore adhesion is a hallmark of metastatic potential in advanced ovarian cancer and an understanding of this process and its response to inhibitors are of clinical relevance.

Integrins are ECM receptors that play a key role in cell-cell and cell-ECM interactions [645]. As such they can function in cell adhesion, migration and invasion [366]. The β1 and β4 integrin subunits have been found to be highly expressed in ovarian cancer cells [689] a fact that was confirmed by the fluorescent micrographs presented in this thesis. Unfortunately experimental problems with respect to the β1 antibody and time and resource constraints meant that the study focused primarily on the β4 integrin subunit. The β4 integrin subunit is thought to play crucial roles in the formation of stable adhesion complexes that affix cells to the basement membrane [352] and secondly, in affecting the PI3K cell signalling pathway by forming a complex with HER-2 receptor tyrosine kinase thereby promoting its signalling [373, 685]. Cancer cells harbouring mutations in *P53* are able to utilise the subunit to promote

cell survival and evade apoptosis [690]. It has strong basal expression in all epithelial tumours [737] and this correlates with poor prognosis and disease spread in breast, pancreas, head and neck carcinomas and ovarian cancers [352]. Furthermore, it has been shown to be significantly overexpressed in SKOV-3 ovarian cancer cell lines [694, 695].

In the cell adhesion experiments, it was noted that canertinib and PHA665752 inhibited the adhesion of OVCAR-5 and SKOV-3 clusters and compact aggregates to collagen-gel matrix (which also contains laminin) without compromising the viability of cells. The combination of the two TKIs was more effective in inhibiting cellular adhesion than the tetra peptide RGDS which inhibits integrin ECM interactions. There was no additive effect on cell adhesion after combining the TKIs and RGDS. Given that most of the cells had already lost adhesion those that remained may represent a subpopulation of cells that are resistant to the inhibitors/RGDS and that may possibly up regulate some other integrin or indeed some other cell-ECM adhesion protein. To the best of my knowledge, there are no previous reports of canertinib or PHA665752 affecting cell adhesion in ovarian cancer cell clusters and aggregates. In other systems, PHA665752 has been shown to affect HGF-mediated adhesion of myeloma cells to fibronectin [700].

The loss of cell adhesion was accompanied with the reduced expression of the receptor tyrosine kinases, but not of the β4 integrin subunits. This may confirm the reports that indicate the association of these integrin subunits with EGFR, HER-2 and c-MET tyrosine kinase receptors [29, 372, 658, 684, 738, 739]. These results provide an impetus for future studies on the efficacy of combinations of small tyrosine kinase inhibitors in preventing cellular adhesion in these ovarian cancer cell lines. The crosstalk between the integrin subunits and EGFR, HER-2 and c-MET is well documented [710] and the phosphorylation of receptor tyrosine kinases has been shown to be associated with integrin signalling [711].

To investigate the possible mechanisms underlining the loss of adhesion, I looked for co-localisation of EGFR and HER-2 with β4 integrin subunits. In the presence of the inhibitors there was a decrease in the co-localisation of EGFR and HER-2 with the β4 integrin subunits. This may underlie the decrease in cell adhesion in the presence of the inhibitors. However, given that this is a preliminary finding and due to time and resource constraints was not able to be investigated further, more investigations are recommended, for example to determine

the expression of other downstream signalling molecules including Akt. Further investigation of the co-localisation of c-MET with the β4 subunit is also needed. The role of the β1 integrin subunits and their possible co-localisation with EGFR, HER-2 and c-MET is also of interest. It is possible to speculate that the reduction in the co-localisation of the receptors and integrin subunits was due to the effect of the inhibitors on the downstream signalling molecules, Akt and ERK, during the four hours of the adhesion assay. Further work including immunoblotting and immunofluorescence analyses addressing the expression and phosphorylation of these molecules may clarify this point.

In summary, this thesis explored the effects of two inhibitors of receptor tyrosine kinases cantertinib and PHA665752 on two ovarian cancer cell lines. These compromised cell growth, cellular metabolism, and the expression and phosphorylation of EGFR, HER-2 and c-MET in 3D cell cultures. They also inhibited VEGF secretion. The inhibitors also affected cell adhesion to a collagen matrix gel possibly due to a decrease in receptor expression which affected co-localisation with integrins. The inhibitory effects on growth and metabolism were compromised when ascitic fluids were included in the assay mixture and it is possible that high levels of proteins in the ascitic fluids may affect the ability of the drug(s) to freely enter the cells. Cell growth and metabolism was enhanced in the presence of the ascitic fluid.

This thesis provides a foundation for further investigations of a combinatorial approach using these inhibitors in the treatment of advanced ovarian cancer. The treatments seem promising in their effects reducing the cellular growth, metabolism, proliferation and adhesion of ovarian cancer cellular clusters and compact aggregates in the *in vitro* environment. However, further work on their effects in an *in vivo* microenvironment is warranted given the results with the ascitic fluid.

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Appendix

Patient 1
-Subtype: High grade papillary Serous
- Cancer stage: IIIC
-No chemo received when ascitic fluid was collected

Patient 2
-Subtype: High grade papillary Serous
-Cancer stage: IC
- Carboplatin+taxol+gemcitabine before ascitic fluid collected

Patient 3
-Subtype: High grade papillary Serous
-cancer stage: IIIC
-No chemo received when ascitic fluid collected